The Influence of T Helper 1 Cell Cytokines on the Regulation of the Transcription Factor FOXP3

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Summary

Impaired functional activity of T regulatory (Treg) cells results in an increased susceptibility to autoimmune diseases and has been reported in allergic patients. The deficiency for Treg-mediated regulation of the pro-allergic effector T cells results in uncontrolled immune reaction against otherwise innocuous antigens. This lack of regulation underlines the critical role of Treg cells in preserving immune homeostasis by maintaining tolerance to self and innocuous antigens.

Treg cells require the transcription factor FOXP3 for both their development and function. Mutations in the *foxp3* gene impair the development of Treg cells and cause a condition known as immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome (IPEX). This illness is characterized by severe autoimmune phenomena including autoimmune enteropathy, dermatitis, thyroiditis, and type 1 diabetes. Given the importance of FOXP3 in the generation and maintenance of Treg cells, understanding the molecular mechanisms regulating its expression is of great interest and provides new perspectives to control autoimmune diseases and allergies.

Although TGF-β signalling allows FOXP3 expression in naïve $CD4^+$ T cells upon activation, little is known about the molecular mechanisms regulating *foxp3* gene expression. This thesis describes a mechanism by which the STAT1-activating cytokines, IL-27 and IFN-γ, amplify TGF-β-induced FOXP3 expression. We identified STAT1-binding elements within the proximal part of the FOXP3 promoter, which we previously hypothesized to function as a key regulatory unit. Direct binding of STAT1 to FOXP3 promoter increases its transactivation by modulating chromatin availability, which increases FOXP3 expression. We reveal that the Th1 cytokines IFN-γ and IL-27 are potentiating the induction of TGF-β-induced Treg cells. This study provides a new understanding of molecular mechanism regulating FOXP3 expression and is

of considerable significance for the elaboration of therapies aiming to restore tolerance by regulatory mechanisms.

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Zusammenfassung

Eine verminderte Funktion von regulatorischen T (Treg) Zellen führt zu einem erhöhten Auftreten von Autoimmunerkrankungen und ist darüber hinaus bei allergischen Patienten bekannt. Hier bedingt eine fehlende Treg-vermittelte Suppression der pro-allergischen Effektor-T Zellen eine unkontrollierte Immunreaktion gegen normalerweise harmlose Antigene. Diese mangelnde Regulierung der Immunantwort unterstreicht die entscheidende Rolle von Treg Zellen, Toleranz zu körpereigenen und harmlosen Antigenen aufrechtzuhalten und dadurch das Immunsystem im Gleichgewicht zu halten.

Treg Zellen benötigen den Transkriptionsfaktor FOXP3 sowohl für ihre Entwicklung als auch für ihre Funktion. Mutationen im *foxp3* Gen führen zu einer unvollständigen Entwicklung der Treg Zellen und verursachen das sogenannte X-chromosomal vererbte Syndrom mit Immundysregulation, Polyendokrinopathie und Enteropathie (IPEX). Diese Krankheit äussert sich durch schwere autoimmune Erkrankungen wie autoimmuner Enteropathie, Hautentzündungen, Thyroiditis und Diabetes Typ 1. In Anbetracht der entscheidenden Rolle von FOXP3 in der Generierung und Aufrechterhaltung von Treg Zellen ist das Verständnis der molekularen Mechanismen, welche die FOXP3-Expression regeln, von großem Interesse und stellt neue Perspektiven dar, um Autoimmunerkrankungen und Allergien zu kontrollieren.

Obwohl TGF-β die FOXP3-Expression in naiven CD4 T Zellen nach deren Aktivierung induziert, ist wenig über die molekularen Mechanismen bekannt, welche die *foxp3*-Genexpression regeln. Die vorliegende Dissertation beschreibt einen Mechanismus, durch den die STAT1-aktivierenden Zytokine IL-27 und IFN-γ die TGF-β-induzierte Expression von FOXP3 verstärken. Wir identifizierten STAT1-bindende Elemente innerhalb des proximalen Teils des FOXP3 Promotors, welcher gemäß unserer vorausgehenden Hypothese eine Schlüsselrolle in der Regulation spielt. Die direkte Bindung von STAT1 an den FOXP3 Promotor führt zu einer Veränderung der Chromatin-Struktur, was die FOXP3-Expression verstärkt. Wir zeigen, dass die Th1-spezifischen Zytokine IFN-γ und IL-27 die Induktion von TGF-β induzierten Treg Zellen

vervielfachen. Diese Studie führt zu einem neuen Verständnis der molekularen Mechanismen, welche die FOXP3-Expression regeln und ist damit für die Entwicklung von Therapien mit dem Ziel, Toleranz durch regulatorische Mechanismen wiederherzustellen, von beträchtlicher Bedeutung.

Abbreviations

AICD	Activation induced cell death
AIDS	Acquired immunodeficiency syndrome
AIRE	Autoimmune regulator
AML1	Acute myeloid leukaemia 1
AP-1	Activator protein 1
APC	Antigen-presenting cells
APECED	Autoimmune polyendocrinopathy-candidiasis-ectodermal-dystrophy syndrome
BRG1	Brahma-related gene 1
CCR6	Chemokine C-C motif receptor 6
CD	Cluster of differentiation
ChIP	Chromatin immunoprecipitation
cTEC	Cortical thymic epithelial cells
CTLA-4	Cytotoxic T lymphocyte antigen 4
DC	Dendritic cell
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
EAE	Experimental autoimmune encephalomyelitis
EBI3	Epstein–Barr-virus-induced gene 3
FKH	Forkhead
FOXP3	Forkhead box p3
GATA-3	GATA-binding protein 3
GFP	Green fluorescent protein
GITR	Glucocorticoid-induced tumor necrosis factor receptor family-related receptor
GP	Glycosylphosphatidylinositol
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
iDC	Immature dendritic cell
IFN	Interferon
IL	Interleukin
IPEX	Immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome
IRF	Interferon regulatory transcription factor
iTreg	Inducible Treg cell
Ku70/Ku80	70 /80 kDa subunit of Ku antigen
L	Ligand
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MBD3	Methyl-CpG binding domain protein 3
mDC	Mature dendritic cell
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
mTEC	Medullary thymic epithelial cell

NFAT	Nuclear factor of activated T cell
NK	Natural killer
NKT	Natural killer T cell
PAMP	Pathogen-associated molecular patterns
PD-1	Programmed death-1
PI3K	Phosphoinositide-3 kinase
PMA	Phorbol 12-myristate 13-acetate
Poly I:C	Polyinosinic and polycytidylic acid
R	Receptor
RAG	Recombination-activating genes
RNA	Ribonucleic acid
ROR	Retinoic orphan receptor
RUNX1	Runt-related transcription factor 1
SBE	Smad binding element
siRNA	Small interfering RNA
SP	Single positive
SP-1	Stimulating protein-1
STAT	Signal transducer and activator of transcription
T-bet	Th1-specific T box transcription factor
TCR	T cell receptor
TGF-β	Transforming growth factor-β
Th	T helper cell
TIEG1	TGF-β-inducible early protein gene 1
TIP60	Tat interacting protein, 60kDa
TLRs	Toll-like receptors
Treg	T regulatory cell
TSA	Tissue-specific antigen
TSLP	Thymic stromal lymphopoetin
TSS	Transcription start site
WB	Western blot

Introduction

1.1 The immune system

The human organism continuously encounters a diversity of potentially harmful pathogens. In response to this constant threat, the human immune system has elaborated a sophisticated anti-pathogen organization involving 2 interacting parts; the innate and the adaptive immunity (Figure 1). Via physical and chemical barriers, the innate immune system represents the first line of host response to pathogen invasion. The cells of the innate immune system interact with pattern recognition receptors and are responsible for the recruitment of immune cells to sites of infection and inflammation. They also activate the complement cascade to identify bacteria and promote clearance of dead cells or antibody complexes. By secreting cytokines or chemokines, they activate the adaptive immune system through a process known as antigen presentation. The specific functions of the adaptive immune system are mediated via responder cells, the T and B lymphocytes. Antigen-mediated triggering of T and B cell receptors initiates specific cell-mediated and humoral immune responses characterised by cytokines or antibody production respectively. The rearrangement of the variable, diversity, and junction gene segments of their receptors generates a repertoire, which is sufficiently diverse to recognize the antigenic component of potential pathogen or toxin [1].

Both innate and adaptive immunity depend on the ability of the immune system to distinguish between harmless self (e.g. components of an organism's body) and non-self pathogenic molecules (e.g. foreign substances). In the healthy individual, the immune system is delicately balanced between self-antigen-driven tolerance and pathogen-driven immunity. A disturbance of this equilibrium results in pathological conditions and severe diseases. The lack of an immune response provokes recurring and life-threatening infections. Immunodeficiency can be the result of a genetic disease, such as severe combined immunodeficiency, or be produced

by pharmaceuticals or infections, such as the acquired immune deficiency syndrome (AIDS) caused by human immunodeficiency virus. An inappropriate response against self leads to autoimmunity. Common autoimmune diseases include rheumatoid arthritis, diabetes mellitus type 1 and lupus erythematous. An excessive response is characterized by an immune reaction against an innocuous antigen. This is seen during allergic reactions, and can cause fatal consequences.

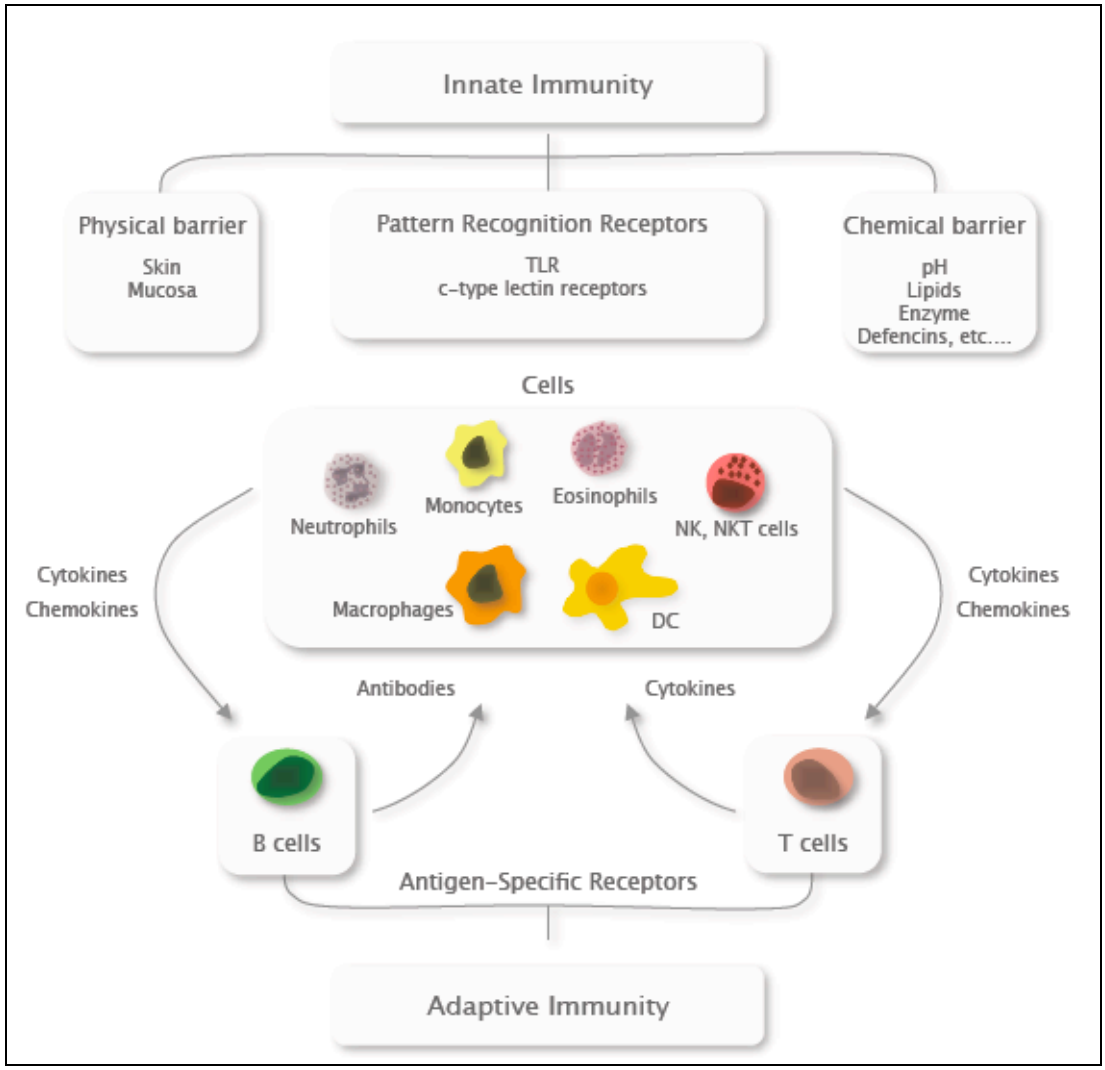

Figure 1: The interactions between innate and adaptive immune system

The cells of the innate immune system secrete cytokines and chemokines that activate cells from the adaptive immune system. NK: Natural Killer cells, NKT: Natural Killer T cells, DC: Dendritic cells.

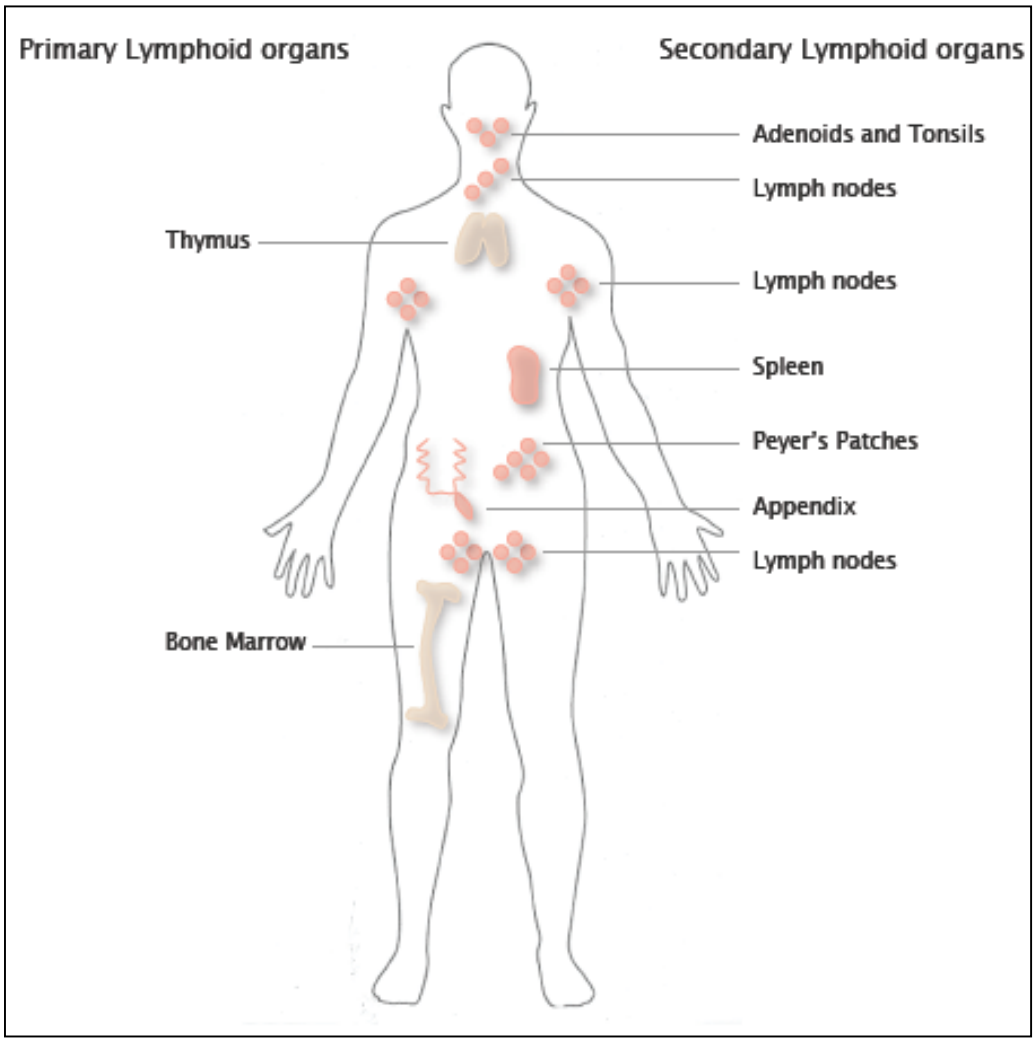

Figure 2: Primary and secondary lymphoid organs

The immune system's organs are called lymphoid organs and they contain a high number of lymphocytes. Interactions between immune and non-immune cells are critical for lymphocyte development and initiation of the immune response. The primary lymphoid organs are the sites for the lymphopoiesis, the cellular differentiation of lymphocytes. Both B and T cells are generated in the bone marrow, but only T cell precursors leave the bone marrow for the thymus, which provides the suitable environment for T cell ontogeny. B cell development occurs in the bone marrow. The peripheral, secondary lymphatic tissues are responsible for T and B cells' commitment into specialised subtypes. Those organs comprise spleen, tonsils, lymph vessels, lymph nodes, adenoids, and skin (Figure 2).

1.2 Decision making during an immune response

Dendritic cells have a pivotal role in the interrelationship between innate and adaptive immunity [2]. Upon encountering a pathogen in peripheral tissues, the immature dendritic cells (iDC) undergo a maturation step allowing them to increase their costimulatory activity, antigen processing, and major histocompatibility complex (MHC) molecule expression while migrating to the lymph nodes. [2]. Secondary lymphoid organs are the key sites for the antigen-driven lymphocyte differentiation. This process requires recognition of peptide antigens presented by MHC molecules and costimulatory molecules expressed on the same antigen presenting cells (APC). For the activation of naïve T lymphocyte to successfully induce an immune response, costimulatory signals are necessary. First, the T cells are activated by the engagement of their surface receptor (TCR), which ensures antigen specificity and MHC restriction of the response. Synergistic signalling by costimulatory molecules is also necessary to sustain and integrate TCR signalling to stimulate optimal T cell proliferation and differentiation [3]. Cytokine production by pathogen-activated immune cells is also needed and direct the type of immune response by orchestrating lineage specific transcription factor and cytokine expression. The differentiation of $CD4^+$ naïve T cells into clonally restricted effector T cells is characterized by the acquisition of a T helper (Th) subset-specific cytokine profile. Figure 3 to 5 depict the events occurring during the naïve $CD4^+$ T cells differentiation into Th1, Th2 or Th17 cells.

1.2.1 Th1 cells

Type 1-associated pathogens include viruses and bacteria. In response to those pathogens, activated APCs produce IL-12, which was identified as the main Th1-inducing factor. Expressing low levels of IL-12 receptor β2 (IL-12Rβ2) at the resting state, naïve $CD4^+$ T cells require TCR, IL-27 or IFN-γ stimulation to increase their IL-12 responsiveness. APC-produced IL-12 also favours the recruitment of IFN-γ producing NK cells. Both IL-27 and IFN-γ are STAT1-activating cytokines and their receptors are expressed on naïve cells [4-6]. STAT1 signalling induces the Th1-specific T box transcription factor (T-

bet), which is the master regulator of Th1 differentiation [7,8] and interferon regulatory factor 1 (IRF1) transcription factor [9]. In addition to the negative regulation of the Th2 specific transcription factor GATA-binding protein 3 (GATA-3) [10], T-bet potentiates expression of the *ifnγ* gene and up-regulates the IL-12Rβ2, whereas IRF1 directly regulates the IL-12Rβ1 [9]. This enables the formation of the IL-12R complex, which is coupled to the Jak2 and Tyk2 kinases and the transcription factor STAT4. Activation of the IL-12R complex further stimulates IFN-γ production and induces expression of IL-18Rα, thereby conferring IL-18's responsiveness to mature Th1 cells. IL-18 serves as a cofactor for IL-12-induced Th1 development and enhances IFN-γ production from effector Th1 cells. Th1 cells express IFN-γ and lymphotoxin-α (LT-α) and mobilize the cellular arm of the immune system. Excessive Th1 responses are associated with various autoimmune and inflammatory disorders (Figure 3).

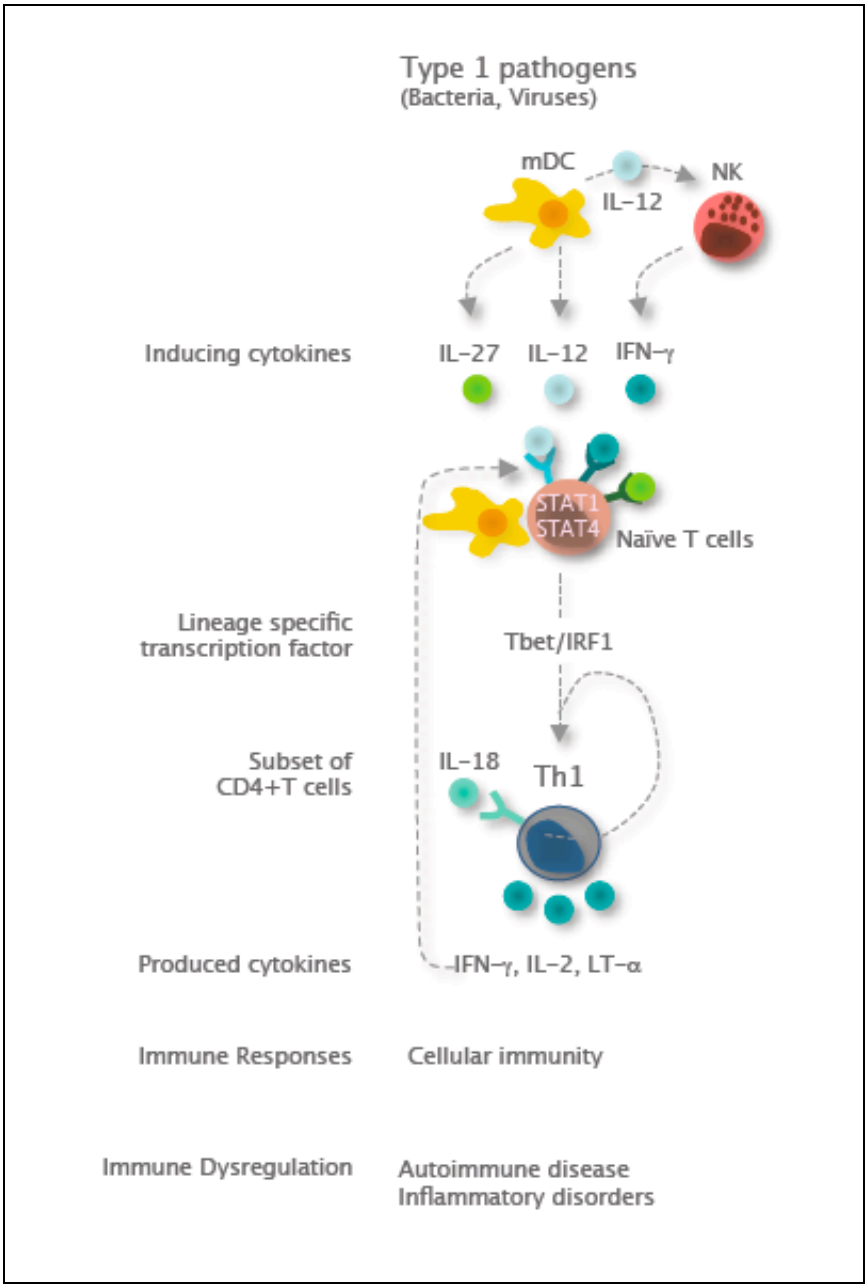

Figure 3: Overview of Th1 cell differentiation

The type 1-associated pathogens induce IL-12, IL-27 and IFN-γ production from innate immune cells (e.g. mDC and NK cells). Those cytokines activate STAT1, STAT4, T-bet and IRF-1, which leads to the establishment of Th1 phenotype, which includes up-regulation of IL-18R and IFN-γ, IL-2 and LT-α production. IL-18 and IFN-γ stimulation further participates in induction of IFN-γ-producing cells.

1.2.2 Th2 cells

Helminths are characterized as type 2-associated pathogens. However, it is unclear how they instruct DCs to produce Th2-inducing factors. *In vitro* studies identified IL-4 as the main Th2-driving cytokine [11], but its contribution *in vivo* is unclear. The cellular source of IL-4 *in vivo* is the subject of considerable debate, but basophils, eosinophils, mast cells and NKT cells, are an important source of IL-4 and have been proposed to be important for Th2 cell differentiation [12,13]. Recently, basophils have been shown to be recruited into draining lymph nodes when stimulated by allergens with enzymatic activity and to produce Th2 promoting factors [14]. The thymic stromal lymphopoietin (TSLP) has also been linked to Th2 differentiation, but whether it acts on DCs or T cells, and whether its involvement is IL-4-dependent or independent is controversial [15]. Th2 differentiation is initiated by signals that originate from the TCR and IL-4 receptors and act cooperatively to up-regulate low-level expression of GATA-3, the master regulator of Th2 differentiation, in a STAT6-dependent manner [16-18]. GATA-3 auto-activates its own expression and drives epigenetic changes that enable expression of the Th2 cytokine cluster (*il4, il5*, and *il13* genes), while suppressing factors critical to the Th1 pathway, such as STAT4 and the IL-12Rβ2 chain [19-21]. Thus, early IL-4 signalling rapidly initiates positive and negative feedback loops that serve to reinforce early commitment to Th2 development, while extinguishing Th1 development. Th2 cells secrete cytokines, including IL-4, IL-13, and IL-5, which are essential for optimal antibody production and elimination of extracellular pathogens. Deregulated production of Th2-derived cytokines, such as IL-4, IL-5 and IL-13, results in immunopathology and is critical for driving asthmatic pathogenesis. [22,23] (Figure 4).

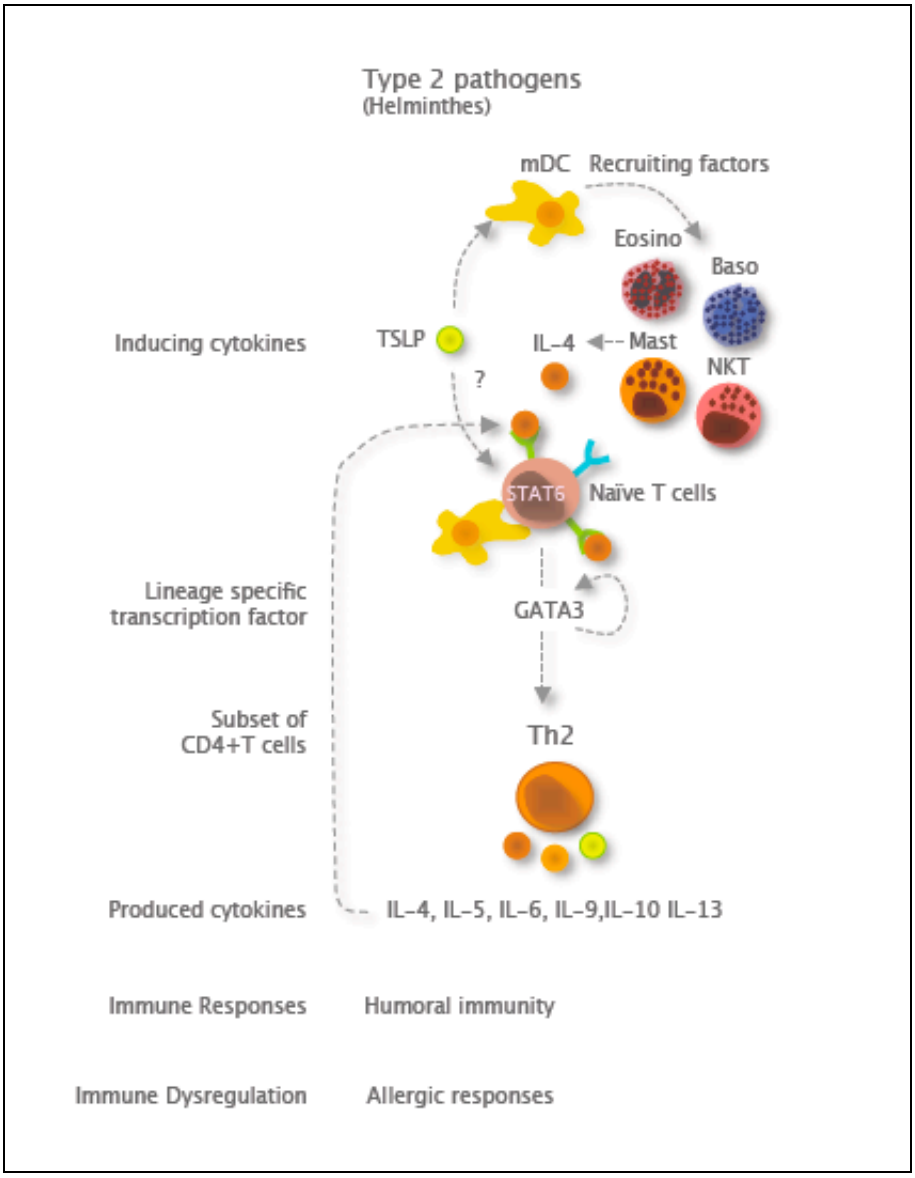

Figure 4: Overview of Th2 cell differentiation

The type 2-associated pathogens induce the recruitment of IL-4-producing cells (e.g. Eosinophils, Basophils, Mast cells and NKT cells). TSLP is linked to Th2 differentiation, but whether it acts on DC or T cells, and whether its involvement is IL-4-dependent or independent is controversial. IL-4 activates STAT6, which induces GATA-3 expression and leads to the establishment of Th2 phenotype. Th2 cells produce many cytokines and participate in hummoral immunity.

1.2.3 Th17 cells

Th17 cells are specialized for enhanced host protection against extracellular bacteria and some fungi. Recently discovered, the requirement for their differentiation is not totally elucidated. Although the differentiation of human and murine Th17 cells may not be identical, there seems to be a consensus that when IL-6 is produced in concert with TGF-β, they act together on naïve $CD4^+$ T cells, via STAT3, to induce the expression of the retinoic orphan receptor (ROR)-γt also designated as RORC2. This Th17 cell specific transcription factor up-regulates the expression of IL-23R and allow the cells to become competent for IL-17A, IL-17F and IL-22 production [24,25]. Human Th17 cells may also produce IL-26 [26], whereas murine Th17 cells have been shown to produce TNF-α and IL-6 [27]. Expression of the IL-23R [26,28] and the chemokine receptor CCR6 [28-32] additionally define this subset. IL-17 is present in multiple chronic inflammatory responses including angiogenesis, recruitment of inflammatory cells, and induction of pro-inflammatory mediators by endothelial and epithelial tissues [33]. Many immune responses that were thought to be Th1 immune responses are now described as Th17 cell-mediated responses. Experimental autoimmune encephalomyelitis (EAE) is an example. In fact, IL-12 and IFN-γ were found to suppress IL-17 production, and the lack of this suppression in IL-12- or IFN-γ-deficient mice contributes to EAE exacerbation [27]. Furthermore, passive transfer of IL-17-producing activated memory $CD4^+$ T cells induced EAE, which confirmed a role of Th17 cells for driving autoimmune inflammation [27] (Figure 5).

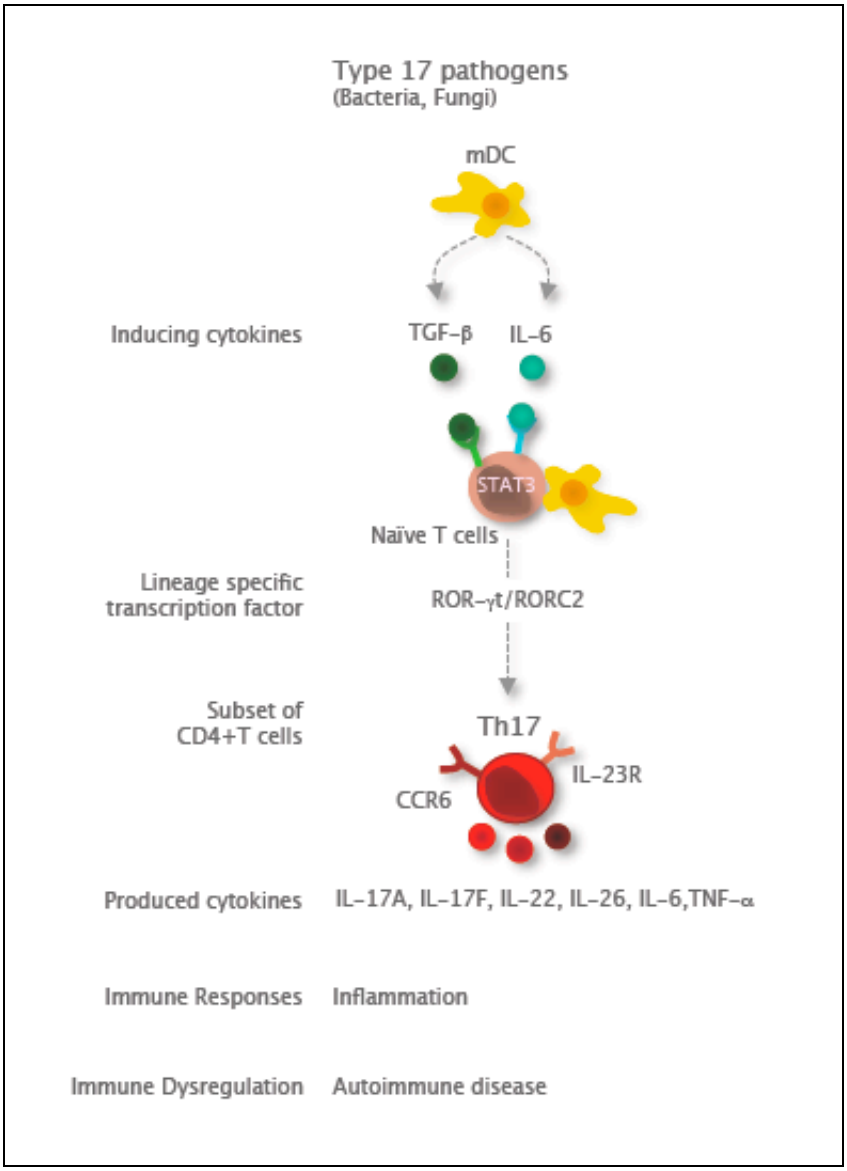

Figure 5: Overview of Th17 cell differentiation

The type 17-associated pathogens induce the production of IL-6 and TGF-β by DC. Via the activation of STAT3, ROR-yt (that is also named RORC2) is activated and participates in the establishment of Th17 phenotype. The cells up-regulate CCR6 and IL-23R and become competent for IL-17A, IL-17F,IL-22, IL-26, IL-6 and TNF-α production.

1.3 Concept of Immune Tolerance

The mammalian immune system possesses the remarkable capacity to endure continued subjection to self-antigens. Imposition and regulation of this self-tolerance within the T cell repertoire is exerted at 2 levels. Central tolerance refers to the deletion of developing autoreactive T cells that bind with high-affinity to intrathymic self-antigens [34,35]. Despite this stringent selection, autoreactive T cells can evade the thymus. Peripheral regulatory mechanisms are then required for the prevention of autoimmunity [36-39]. This requirement to prevent excessive activity led to rapid acceptance of a T suppressor or Treg cell population. Treg cells may suppress the activation of those self-reactive T cells that escape selection [39,40].

The importance of central and peripheral tolerance to immune health has been shown in recent years with the understanding of the molecular basis of 2 inherited autoimmune syndromes. In both cases, the affected molecules are transcriptional regulators: the autoimmune regulator (AIRE) and the forkhead box P3 (FOXP3). Mutations in the *aire* gene lead to defective clonal deletion of T cells and to the multi-organ syndrome known as autoimmune polyendocrinopathy-candidiasis-ectodermal-dystrophy syndrome (APECED) [41]. Mutations in the *foxp3* gene impair the development of Treg cells and cause the syndrome known as immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome (IPEX) [42]. The association between autoimmune diseases and mutations at particular genetic loci suggests a causal relationship.

1.3.1 Central Tolerance

The random generation of receptor specificities during T cell differentiation increases the potential to respond to self-proteins, which would result in autoimmune tissue damage. To bypass this problem, one approach is to test T cells for self-reactivity during their maturation in the thymus and to delete those posing the greatest menace. Thus, central tolerance represents the

mechanism by which newly developing T cells are selected for lack of high reactivity to self (Figure 6). Thymocyte progenitors mature in thymus from double negative (DN) 1 to DN4 stage. The random production of TCR during their differentiation towards the double positive (DP) stage generates many unsuccessful TCR.

Positive selection is a crucial step that enriches for MHC restricted T cell progenitors by allowing only cells that express a TCR that can interact with self-peptide-MHC complexes to further differentiate. Immature DP progenitors can be positively selected when they encounter an appropriate self-peptide-MHC complex on cortical thymic epithelial cells (cTECs) [43].

Negative selection is necessary to eliminate potentially autoreactive T cells generated during positive selection. Positively selected DP thymocytes migrate towards the medulla and interact with medullary thymic epithelial cells (mTECs) that express costimulatory molecules and peripheral tissue-specific antigens (TSAs) under the control of the transcriptional regulator AIRE [44, 45]. DCs are also present in the medulla. They express costimulatory molecules, such as CD40, CD80, and can cross-present TSAs that are produced by mTECs to developing T cells and thereby induce clonal deletion.

The main mechanism of negative selection is clonal deletion, but receptor editing and anergy have also been described, whereas these are thought to have a minor role. These 3 processes impair or eliminate high-affinity self-reactive thymocytes. All are thought to be induced by high affinity self-peptide-MHC interactions with TCR in the thymus [46]. It was reported that a mechanism selecting for high-affinity self-reactive cells results in differentiation into a Treg cell phenotype [47-49]

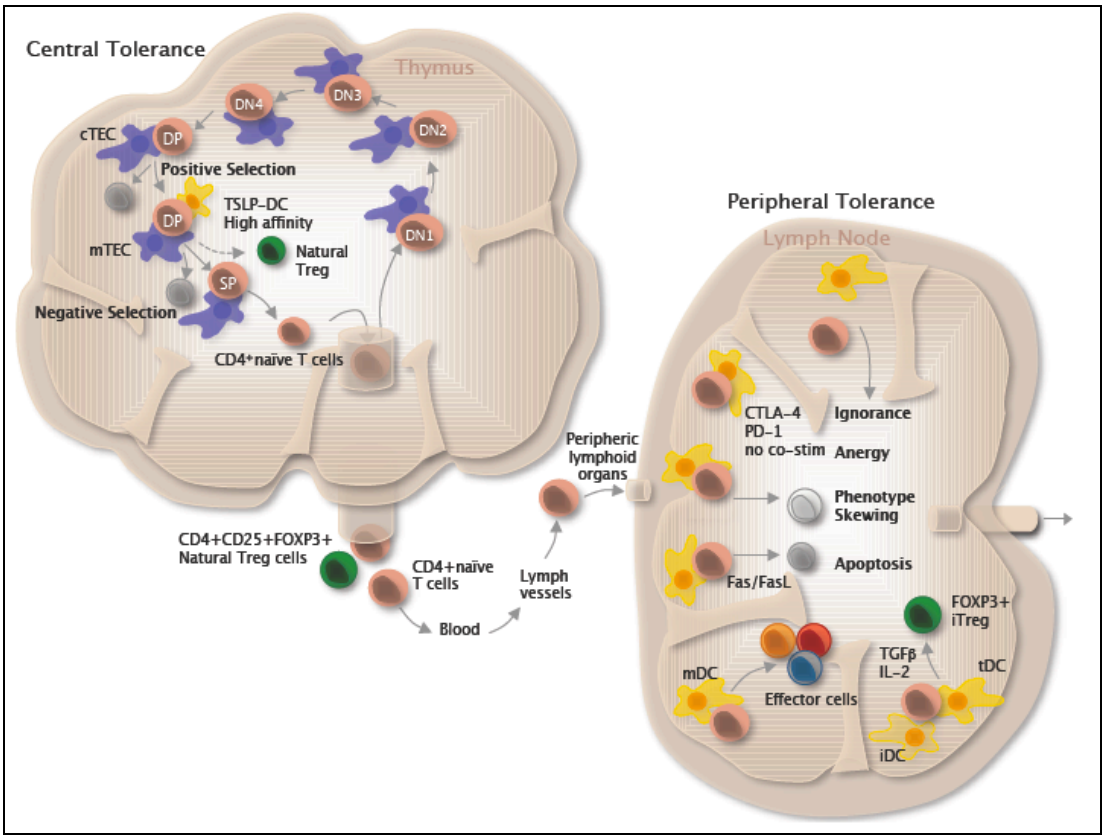

Figure 6: Central and Peripheral tolerance mechanisms.

During the development of T lymphocytes in the thymus, central tolerance mechanisms eliminate most of the self reactive T cells. Peripheral Tolerance mechanisms in the peripheric lymphoid organs are necessary to complete this elimination.

1.3.2 Peripheral Tolerance

Although central-tolerance mechanisms are efficient, they are unlikely to eliminate all self-reactive lymphocytes. Low affinity TCR for self-antigens have been found in healthy individual in the periphery [50-52], which is consistent with the predominant deletion of high-affinity cells in the thymus. Peripheral tolerance mechanisms have been invoked to explain the absence of auto-reactivity toward tissue-restricted self-constituents. Peripheral tolerance can act at several levels using mechanisms acting either directly on the self-reactive T cell, named T cell intrinsic, or indirectly via additional cells,

designated T cell extrinsic. T cell intrinsic mechanisms comprise ignorance, anergy, phenotypic skewing and apoptosis, whereas T cell extrinsic mechanisms include tolerogenic DC and Treg cells (Figure 6).

1.3.2.1 T cell intrinsic mechanisms

Four T cell intrinsic mechanisms are known to play a role in immune tolerance: ignorance, anergy, phenotype skewing and apoptosis. The concept of ignorance involves anatomic separation. By hiding self antigens behind a tissue barrier impermeable for circulating immune cells, self-reactive T cells might never encounter the self-protein they recognize, and therefore exist in a state of ignorance. Anergy was first described as the result of TCR ligation in the absence of costimulation [53,54]. However, later studies showed that anergy possibly involves interaction with inhibitory molecules, which are induced after encountering self-proteins. The T cell inhibitory molecules consist of the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) [55] and the programmed death-1 (PD-1) [56,57]. Phenotype skewing refers to a full activation of the T cells interacting with self-protein. In this case, self-reactive T cells become activated but fail to induce autoimmune tissue damage because of impaired lymphocyte trafficking. Finally, self-reactive T cells might undergo apoptosis following contact with self-protein by activation-induced cell death (AICD) involving up-regulation of T cell Fas ligand and subsequent signalling through the death receptor Fas [58].

1.3.2.2 T cell extrinsic mechanisms

DCs have emerged as the key APCs involved in process initiating immune responses and inducing T cell tolerance. Whether any immature DC can perform a tolerogenic function, or whether this task is mediated by a distinct lineage of DCs is unknown. Following pathogen exposure, DCs undergo a maturation process, which modifies their antigen-processing capacity and their surface phenotype [1]. Their capacity to activate T cells is gained by up-regulating costimulatory molecules. Consequently, the ability of DCs to initiate

immune responses would depend on encountering a pathogen-associated molecule [59]. DCs constantly take up self-proteins and present them to self-reactive T cells. In the absence of a pathogen trigger, the low expression of costimulatory molecules by those immature DCs or by a distinct subset of 'tolerogenic' DCs, leads to T cell tolerance rather than activation [38]. It is possible that immature or 'tolerogenic' DCs maintain T cell tolerance indirectly by the induction of Treg cells [60], a distinct subset of T cells with regulatory functions.

1.3.3 T regulatory cells

T cells were shown to possess, in addition to their effector and positive co-operative functions, a role in depressing immune responses [61-71]. These previously named suppressor T cells are now termed Treg cells. They can mediate their suppressive mechanisms via cell-contact-dependent mechanisms [72], inhibitory cytokine production (IL-10 or TGF-β) [73,74] or by consumption of T cell growth factors [75]. In contrast to effector cells that can be characterized by their unique cytokine profile Treg cells do not produce specific cytokines. Even though IL-10 and TGF-β have often been identified as Treg cytokines, they are not Treg cell-exclusive. Recently, IL-35 has been shown to be constitutively and exclusively produced by mouse Treg cells [76]. IL-35 is an heterodimeric cytokine consisting of the Epstein-Barr virus induced gene 3 (EBI3) and the IL-12-p35 subunit. Treg from EBI3-/- and p35-/- mice had reduced regulatory activity both *in vitro* and *in vivo*, which indicated that in mice, the EBI3–p35 complex (IL-35) is required for the suppressive activity of regulatory T cells.

1.3.3.1 Naturally occurring Treg cells

Naturally occurring $CD4^+CD25^+$Treg cells comprise 3%-10% of peripheral $CD4^+$ T cells and are the major population of suppressing T cells maintaining peripheral immune tolerance to self antigens [77]. The natural Treg cells originate from the thymus [49,78] and require the expression of the FOXP3 transcription factor for their development and function [79,80]. How the differentiation of the natural Treg is regulated is not fully understood, but it requires a high affinity self reactive TCR [49]. It was postulated that TSLP instructed DCs positively select Treg cells exhibiting medium to high self-reactive affinity within the medulla of the thymus [81]. CD28-costimulation directly signals DP thymocytes to express FOXP3, as well as glucocorticoid-induced TNF receptor family related receptor (GITR) and CTLA-4, which initiate the Treg cell differentiation program. [82]. Mature Treg can be identified by their constitutive expression of CD25, CTLA-4, and GITR [83-85].

Natural Treg cells that migrate in the periphery keep their phenotype and function, which are essentially cytokine-independent.

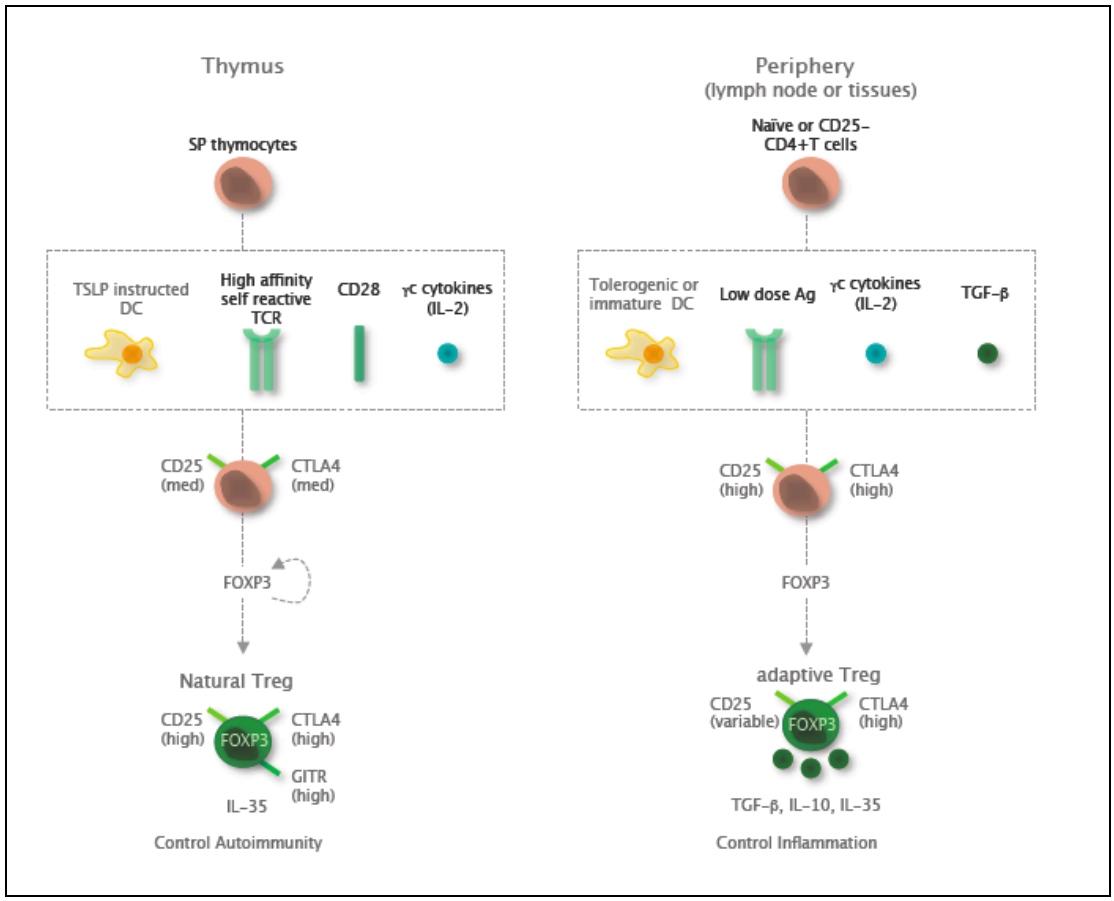

Figure 7: Induction of Treg cells in the thymus and in the periphery

Natural Treg cell differentiation occurs in the thymus whereas iTreg cells differentiation takes place in the periphery. Natural Treg cells originate from high affinity self-reactive TCR bearing SP thymocytes activated by TLSP instructed DC, CD28 or γ chain cytokines. They express high levels of CD25, CTLA-4 and GITR and control autoimmunity. Adaptive or iTreg cells originate from $CD25^-$ precursor or naïve $CD4^+$ T cells. Induced by tolerogenic or immature DCs, low dose of antigen, γ chain cytokines or TGF-β, they expressed variable levels of CD25 and they control inflammation.

1.3.3.2 Inducible or adaptive Treg cells

In parallel to the natural Treg, other subsets of $CD4^+$ Treg cells have been described to successfully control immune responses not only to self-antigens but also to a wide variety of non self-antigens (microbial, tumoral, and transplantation antigens). These $CD4^+FOXP3^+$ Treg cells, termed adaptive or inducible (iTreg), are not present in the thymus and can be generated in peripheral lymphoid tissues from peripheral precursors that are $CD4^+CD25^-$ or from naive $CD4^+FOXP3^-$ progenitors. Two major factors appear to be involved in the generation of functional $FOXP3^+$ Treg in the periphery; the mode of antigen presentation, and the presence of specialized immunoregulatory cytokines. Immature or tolerogenic DCs have also been associated with the generation of Treg cells in the periphery. Low dose of antigen presented by DC seem to be important for adaptive Treg's generation. The role for TGF-β in the induction of Treg cells is now well accepted. TGF-β can induce naive human $CD4^+$ T cells to develop powerful contact-dependent suppressor activity that is mediated by TGF-β or IL-10 [86]. The unique cytokine dependence is one of the main features that differentiate adaptive from natural Treg cells. However, the expression of the transcription factor FOXP3 is common to both subtypes of Treg cells.

1.4 FOXP3: the master regulator of Treg cells

The characterization of the IPEX syndrome and the mapping of the syndrome to the X chromosome greatly improved our understanding of the function of FOXP3. IPEX is a rare disorder caused by mutations in the *Foxp3* gene that result in the defective development of Treg cells and leads to severe autoimmune disease including autoimmune enteropathy, dermatitis, thyroiditis, and type 1 diabetes, frequently resulting in death within the first 2 years of life [87]. FOXP3 belongs to the forkhead (FKH) factor family, containing 3 functional domains: a highly conserved C-terminal winged-helix/forkhead deoxyribonucleic acid (DNA)-binding domain, a C2H2 zinc finger, and a leucine zipper [87]. The N-terminal domain of FOXP3 is unique to the FOXP subfamily, and it is critical for transcriptional repression [87]. With the exception of the zinc-finger motif, mutations have been found in each of the functional domains of the *foxp3* gene in individuals affected by IPEX, which indicates that each of these regions are important for proper FOXP3 function [88-90]. Mutations in the FKH domain of FOXP3 have been shown to affect its ability to bind DNA, whereas 2 separate mutations in the leucine-zipper-like motif have been found to affect the homo- and heterodimerization of FOXP3 [91,92]. Many of the FOXP3 mutations observed in IPEX syndrome are missense mutations in the FOXP3 FKH domain, but mutations have been found across the length of the gene. Mutations in the FKH domain ablate the ability of FOXP3 to inhibit transcription of a reporter construct [91]. Interestingly, some IPEX patients were shown to have single codon mutations in the leucine zipper domain of FOXP3 resulting in a failure to homodimerize and in reduced ability to repress transcription [93,94]. These data strongly suggest that the leucine zipper domain of FOXP3 is critical for proper function. Additional mutations in the amino terminal domain have been identified as critical for FOXP3 function.

1.4.1 The function of FOXP3

It is now well documented that FOXP3 is the key transcription factor for the development and function of Treg cells. [80]. Two recent studies used chromatin immunoprecipitation-on-chip (ChIP-on-chip) technology to identify the targets of FOXP3 and showed that FOXP3 could function both as a transcriptional activator and a transcriptional repressor. Comparison of primary $CD4^+Foxp3^+$ with $CD4^+Foxp3^-$ mouse T cells outlined approximately 700 genes that are regulated by Foxp3 [95]. The use of a T cell hybridoma that either did or did not express an introduced *foxp3* gene underlined that about 1,100 genes are Foxp3 regulated [96]. Both studies found that most of the Foxp3-target genes identified by the ChIP-on-chip analyses were differentially regulated in naturally occurring Treg cells. A more targeted ChIP-on-chip study using human Treg cells showed that CD127 (which encodes the IL-7 receptor α-chain) is a FOXP3-target gene and is negatively regulated by FOXP3 [97].

Although the mechanisms by which FOXP3 produces Treg-cell-mediated suppression of various immune cells are largely unknown, it is clear that the function of FOXP3 is dependent on its binding partners, including several key transcription factors and various corepressors and/or coactivators in regulatory T cells [98]. FOXP3 can be an acetylated protein [99] or an oligomeric component of a large molecular complex [100,101]. One mechanism of FOXP3-mediated transcriptional repression involves direct contact with the nuclear factor of activated T cells (NFAT) and its subsequent inhibition. FOXP3 and NFAT can bind cooperatively to a binding site on the IL-2 promoter and inhibit its expression [102]. The ability of FOXP3 to inhibit transcription was abolished by mutation of the residues in its FKH domain that are predicted to interact with NFAT. Furthermore, FOXP3 actively represses transcription at the chromatin level by recruiting corepressors such as the histone acetyltransferase (HAT), Tat interacting protein 60kDa (TIP60) ,and histone deacetylases (HDAC) 7 that favour a restrictive chromatin conformation [98,99]. In addition to its interaction with NFATc2 [102,103],

FOXP3 has been shown to cofractionate with other transcriptional factors in a low molecular weight complex. [100]. So FOXP3 can interact with acute myeloid leukaemia 1/ runt-related transcription factor 1 (AML1/RUNX1), [104], FOXP1 [101,105], ROR-α [87] or ROR-γt [106]. Other FOXP3-associated chromatin remodelling factors, including brahma-related gene 1 (BRG1), 70 /80 kDa subunit of Ku antigen (Ku70/Ku80) and methyl-CpG binding domain protein 3 (MBD3), cofractionate with nuclear FOXP3 in larger molecular weight complexes [100]. FOXP3-associated chromatin remodelling factors and enzymatic subunits may be affected by diverse signals and may modify FOXP3's target and function.

Several recent papers have addressed the precise role of FOXP3 in Treg cell development and function in the thymus and in the periphery. The use of male mice carrying the Foxp3-green fluorescent protein (GFP) knock-in allele, resulting in a fluorescent non-functional Foxp3 protein, demonstrated that Foxp3 was necessary for induction and maintenance of Treg cell phenotype and function. While retaining many of the cell-surface markers of natural Treg cells, the Foxp3-knockout T cells from those mice lacked suppressor function. However, they regained the ability to proliferate and produce cytokines when stimulated [107,108]. This indicates that Foxp3 is required for the suppressive functions of Treg cells, as well as for their anergic state, but other factors have important roles in Treg cell development. The complete lack of FOXP3 during neonatal development results in fatal autoimmunity. However, a reduction in the level of FOXP3 also leads to the same phenotype [109,110]. This underlines that not only the presence of FOXP3 is important for Treg cell function, but its expression level is also crucial [111,112].

1.4.2 The regulation of FOXP3

Even though FOXP3 is required for the development and function of Treg cells both in the thymus and in the periphery [79,80], relatively little is known about the molecular mechanisms regulating *foxp3* gene expression. A variety

of signals have been described to positively influence FOXP3 expression (Figure 8).

1.4.2.1 The role of TGF-β

TGF-β is a pleiotropic cytokine, which is implicated in the regulation of immune responses [113]. Ablation of either TGF-β or its receptor results in excessive T cell responses and severe autoimmunity [114-116]. TGF-β induces the conversion of naive $CD4^+$ T cells into $FOXP3^+$ Treg cells *in vitro* and is important for the maintenance of Treg cells *in vivo* [114,117-119]. The TGF-β-converted $CD4^+$ T cells exhibited cell-contact-dependent suppressor activity *in vitro* when cocultured with normal $CD4^+$ T cells [117,118]. However, until recently, no specific mechanism had been proposed to explain the TGF-β-induced FOXP3 expression. A positive autoregulatory loop was postulated, in which Smad7, a transcription factor that is normally induced by TGF-β and limits TGF-β signalling, is down-regulated by FOXP3 [118]. This down-regulation of Smad7 renders $CD25^-$ T cells highly susceptible to the further inductive effects of TGF-β signalling via Smad3 and Smad4. Recently, Smad3 and NFAT have been described to cooperate to induce FOXP3 expression by binding and acting on its enhancer [120]. The ubiquitination of the transcription factor TGF-β-inducible early gene 1 (TIEG1) by Itch also increase the transcriptional activation of the FOXP3 promoter [121]. Together, these recent findings propose a molecular mechanism of FOXP3 induction by TGF-β, but further studies are needed to complete our understanding of the regulation of FOXP3.

1.4.2.2 The role of the T cell receptor

TCR triggering is a main feature of naïve cell differentiation and is necessary for the induction of FOXP3. Studies of FOXP3 regulation have revealed a proximal 5' regulatory region of the FOXP3 promoter containing NFAT and AP-1 binding sites and were reported to contribute to TCR-mediated regulation of the *foxp3* gene [122]. Binding of transcription factors stimulating protein-1 (Sp-1), NFAT, activator protein 1 (AP-1) to the FOXP3 promoter

have also been reported and are important for the regulation of the gene [123]. A potent new TCR response element in the *foxp3* gene that demonstrate DNA methylation-dependent control of *foxp3* gene expression, have also been identified [124].

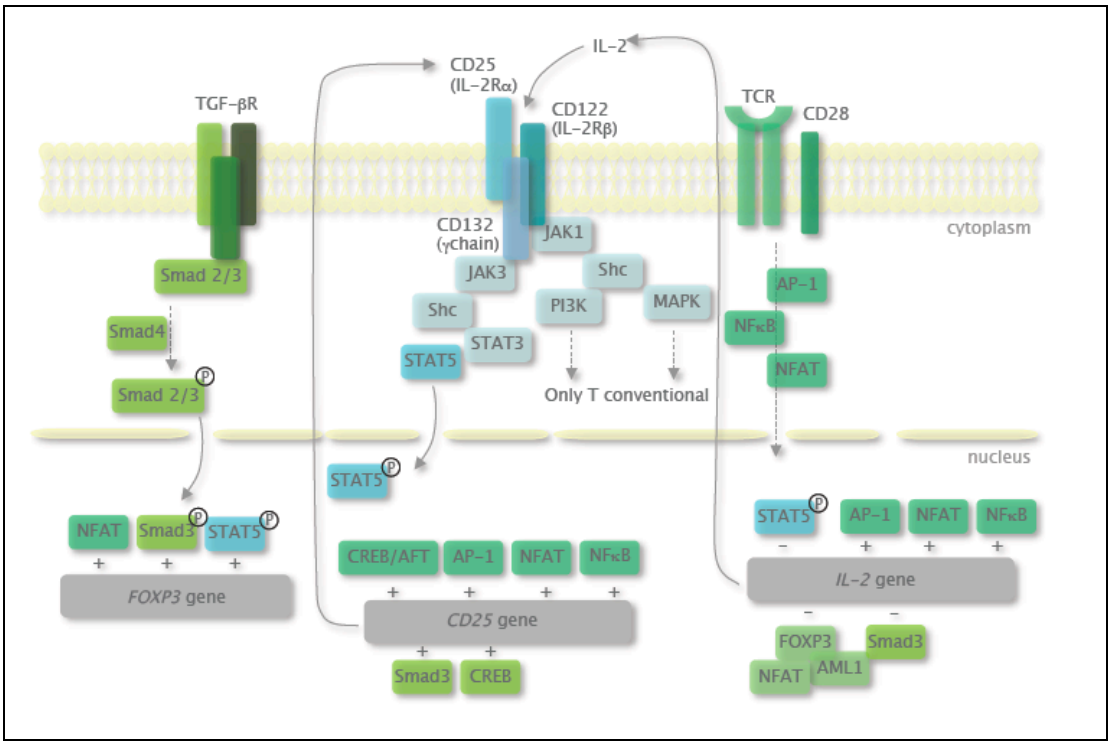

Figure 8: Intracellular pathways controlling Treg cells differentiation

TGF-β, IL-2R and TCR/CD28 act in concert on *foxp3*, *cd25* and *il2* genes to control Treg cell differentiation.

1.4.2.3 The role of IL-2

Early paradigm proposed a central role of IL-2 in protective immune response by supporting effector T cells growth and acting as a mitogenic cytokine. However, much data support an essential role in immune tolerance. IL-2 acts by binding to the high affinity IL-2 receptor consisting of 3 subunits: IL-2Rα (CD25), IL-2Rβ (CD122), and the common gamma chain or γc (CD132). CD25 expression by Treg cells is influenced by TCR activation and IL-2 up-regulation. It was shown that TCR activation and TGF-β signalling also regulate CD25 expression and that the constitutive expression of CD25 by Treg cells might partly be due through their production of TGF-β [125]. The

serious lymphoproliferative and autoimmune disorder observed in IL-2R α or β deficient mice is due to the inability to produce Treg cells [126-129]. In addition to its contribution to the thymic development of Treg cells, by shaping the number of Treg cells and the levels of FOXP3, IL-2 also functions in maintaining Treg cells in peripheral compartments. IL-2 signalling activates mitogen-activated protein kinase (MAPK), phosphoinositide-3 kinase (PI3K) and signal transducer and activator of transcription (STAT) 5 pathway in conventional T cells, whereas STAT5 activation is the main outcome of IL-2R signalling in Treg cells [130-133]. A patient with a homozygous missense mutation in the *stat5b* gene has also been identified with immune dysregulation and impaired expression of FOXP3 and Treg cell function [134], implicating STAT5 activation with human Treg cell production. ChiP analysis shows that STAT5 binds to the FOXP3 promoter [135] and the untranslated region (UTR) of the *foxp3* gene in $CD25^+$ cells [123].

1.4.2.4 The role of other T helper cell subsets

Several GATA-3 binding sites within the FOXP3 promoter negatively regulate FOXP3 expression [136]. IL-4-induced GATA-3 repressed FOXP3 expression directly by binding to the FOXP3 promoter region. A palindromic GATA-site is located 303 bp upstream of the transcription start site (TSS) in the FOXP3 promoter [122]. Site-specific mutation of this site increased the activity of the promoter constructs, thus revealing the repressive nature of this GATA element. Keeping GATA-3 expression at low levels might be required to induce efficient $FOXP3^+$ iTreg cell generation.

Whether Th1-inducing cytokines (e.g. IL-12, IFN-γ and IL-27) regulate FOXP3 expression has not been fully investigated. IFN-γ has been shown to be required for FOXP3 expression and thus, for conversion of $CD4^+$ T cells into Treg cells during EAE [137]. IL-27, which was initially described to control development and activation of Th1 cells, also exerts anti-inflammatory functions *in vivo* and has emerged as an immunoregulatory cytokine [138-

141]. IL-27 is a heterodimeric cytokine, which consists of p28 and EBI3 subunits. The p28 chain is related to IL-12p35 and has a classical cytokine structure, whereas the EBI3 is related to IL12p40 and structurally resembles the soluble IL-6R [4,142]. IL-27 is expressed predominantly by APCs and the production of both subunits can be induced by toll-like receptor ligands such as lipopolysaccharide (LPS), polyriboinosinic:polyribocytidylic acid (Poly I:C) and by intact *Escherichia coli* [4,143,144]. Additionally, signalling via CD40L and IL-1β can up-regulate the EBI3 subunit, whereas p28 has been shown to be up-regulated by IFN-γ [144,145]. IL-27 is a pleiotropic cytokine with contrasting effects, as it also promotes early commitment of naïve T cells to the Th1 cell lineage [5,146] and was recently shown to directly antagonize the development of Th17-cell responses and to limit IL-17-cell-driven inflammation in the central nervous system [140,147]. IL-27 also limits Th17-cell-mediated uveitis and scleritis and was suggested to contribute to immune privilege [142]. Another key function of IL-27 might be to regulate early inflammatory events during acute infections, as IL-27 neutralization protects mice against lethal septic peritonitis by enhancing the influx and oxidative-burst capacity of neutrophils [148].

1.4.2.5 The role of STAT molecules

One mechanism by which cytokines elicit biological responses is through the activation of STAT signalling pathways. Similar to the STAT-mediated regulation of the specific lineage transcription factor T-bet, GATA-3 and RORC2, several studies reported a role for STAT molecules on the *foxp3* gene regulation. IL-2-activated STAT5 can bind to the distal part of the FOXP3 promoter [135] as well as to the UTR of the *foxp3* gene in $CD4^+CD25^+$ cells [123], which positively regulate its expression. STAT3 and STAT5 binding sites have also been found in the exons of the *foxp3* gene [149] and STAT3 small interfering RNA (siRNA) decreases the FOXP3 protein expression [150], indicating a positive regulation of FOXP3 by STAT3. It has been shown that the STAT1 activating cytokine IFN-γ is required for FOXP3 expression and for conversion of $CD4^+CD25^-$ T cells into Treg cells.

Furthermore, impairment of the generation of Treg cells was reported in $STAT1^{-/-}$ mice, demonstrating that STAT1 is implicated in the generation of $CD4^{+}CD25^{+}$ Treg cells [151]. Thus, STAT molecules play a crucial role in the regulation of FOXP3 expression.

Conclusion and aim of the study

The aim of this study was to gain understanding of the regulation of FOXP3, particularly by Th1-inducing cytokines at the promoter level. We identified a new pathway regulating FOXP3 expression. This finding provides a valuable tool for the elaboration of therapy aiming at the regulation of FOXP3 expression.

Material and Methods

2.1 T cell population isolation

Peripheral blood mononuclear cells (PBMC) were isolated from Buffy coats of healthy donors by Ficoll (Biochrom KG) density gradient centrifugation. $CD4^+$ T cells were purified with anti CD4-Dynal magnetic beads and Detach-a-Bead antibodies (Dynal) according to the manufacturer's instructions. $CD4^+CD45RA^+$ cells were purified by MACS (Miltenyi). $CD4^+CD45RA^+$ T cell purity was consistently ≥90% as determined by FACS analysis.

2.2 *In vitro T cell differentiation*

All T cells were maintained in AIM-V serum free medium (Gibco) with 30 ng/ml IL-2 (Peprotech) and polyclonally stimulated with soluble anti-CD3 (2.5 μg/ml; clone Okt3; IgG1) and anti-CD28 (2.5 μg/ml; clone B7G5). Naïve T cells were driven into neutral condition: 5 μg/ml anti-IL-4, 5 μg/ml anti-IL-12 and 1 μg/ml anti-IFN-γ, into Th1 condition: 5 μg/ml anti-IL-4 and 25 ng/ml IL-12 or into iTreg condition: 5 μg/ml anti-IL-4, 5 μg/ml anti-IL-12, 1 μg/ml anti-IFN-γ and 5 ng/ml TGF-β (R&D systems). Proliferating cells were expanded in medium containing IL-2 (30 ng/ml). For stimulation of iTreg cells with Th1 cytokines, IL-27 was used at 60 ng/ml, IFN-γ at 500 U/ml and IL-12 at 25 ng/ml.

2.3 RNA isolation and cDNA synthesis

RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Reverse transcription of human samples was performed with Fermentas reverse transcription reagents (Fermentas) with random hexamers according to the manufacturer's protocol.

2.4 Quantitative real-time PCR

The PCR primers detecting FOXP3 and T-bet were designed based on the sequences reported in GenBank with the Primer Express software version 1.2 (Applied Biosystems), as listed in Table 1. The PCR primers and probes detecting IL-12 p35, IL-12p28 and EBI3 were purchased from Applied Biosystems. The prepared cDNAs were amplified using SYBR-PCR Mastermix (Applied Biosystems), according to the recommendations of the manufacturer in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Relative quantification and calculation of the range of confidence were performed using the comparative ΔΔCT method, as described [125,139]. All amplifications were conducted in triplicates.

Primers	**Sequence**
FOXP3 sense	GAAACAGCACATTCCCAGAGTTC
FOXP3 antisense	ATGGCCCAGCGGATGAG
T-bet sense	GATGCGCCAGGAAGTTTCAT
T-bet antisense	GCACAATCATCTGGGTCACATT
Elongation Factor 1a sense	CTGAACCATCCAGGCCAAAT
Elongation Factor 1a antisense	GCCGTGTGGCAATCCAAT

Table 1 Primers for Real Time PCR

2.5 Flow cytometry

Cells were stained with the mAb CD25 (Beckman Coulter) prior the FOXP3 intracellular staining which was performed according to the manufacturer's protocol using AlexaFluor®488 anti-human FOXP3 Flow kit clone (295D; Biolegend).

Intracellular cytokine labelling was performed with R-phycoerythrin-conjugated mouse anti human IFN-γ monoclonal antibody (clone B27; BD Biosciences). Cells were stimulated with PMA (25 ng/ml), ionomycin (1 mg/ml), and Brefeldin A (10 mg/ml) (Sigma-Aldrich) 5 h prior to staining. Cells were fixed and permeabilized using BD cytofix/cytoperm TM kit (BD Biosciences) according to the manufacturer's instructions. Matched isotype controls were used at the same protein concentration as the respective antibodies.

Treg marker surface staining was performed using fluorescein isothiocyanate-conjugated mouse anti-PD1 antibody (eBioscience) and mouse anti-GITR/TNFRSF18 (R&D systems), R-phycoerythrin-conjugated mouse anti CTLA-4 (CD152) antibody (BD Pharmingen) and mouse anti-CD-103 antibody (Dako). Matched isotype controls were used at the same protein concentration as the respective antibodies.

Cell analysis by flow cytometry was performed using a Four-color FACS EPICS^{TM} XL-MCL (Beckman Coulter) using the software Expo^{TM}32 version for data acquisition and evaluation.

2.6 Cytokine quantification

Supernatants of *in vitro* differentiated cells that were restimulated at day 12 with anti-CD3 (2.5 μg/ml) and anti-CD28 (2.5 μg/ml) were analyzed for cytokines using Bio-Plex™ 200 System according to the manufacturer's protocol (BioRad).

2.7 Suppression assay

$CD4^+CD45RA^+$ T cells were cultured into iTreg condition during 10 days. At that point, autologous PBMC and $CD4^+$T cells were isolated. $CD4^+$ responder T cells were washed twice with PBS and labeled in PBS 2 μM CFSE (Molecular Probes Invitrogen) for 3 min at RT. Cells were washed twice with complete RPMI (Life Technologies). Cells were cultured in 96 round-bottom plates with anti-CD3 (2.5 μg) during 5 days. The proliferation of $CD4^+$ responder T cells was analyzed by flow cytometry.

2.8 Bioinformatics

Transcription factor binding sites were identified using TESS software (<www.cbil.upenn.edu/cgi-bin/tess/tess33>), which uses matrices of the Transfac database.

2.9 Western blotting

Nuclear Extraction was performed as follows: All buffers contained EDTA-free complete protease inhibitor (RocheDiagnostics). Cells were resuspended in 400 μl buffer A (10 mM KCl, 1 mM DTT, 10 mM Hepes pH 7.9, 1 mM EDTA). After 15 min of incubation, 25 μl of 10% Nonidet P-40 (Sigma-Aldrich) was added and the tube was vortexed vigorously for 30 sec followed by a

centrifugation step (1 min, maximum speed, 4 °C). The translucent nuclear pellet was washed once with 1 ml of buffer A, resuspended in 50 μl of buffer B (400 mM NaCl, 1 mM DTT, 20 mM Hepes pH 7.9, 1 mM EDTA), and incubated for 15 min at 4 °C on a roller. Nuclear debris and genomic DNA were removed by centrifugation (5 min, maximum speed, 4 °C). The supernatant was diluted 1/3 with buffer D (20 mM Hepes pH 7.9, 1.5 mM MgCl2, 0.2 mM EDTA, 1 mM DTT, 0.1 % Nonidet P-40). Total protein concentration was determined by a colorimetric protein assay (BioRad). Samples were loaded next to a protein-mass ladder (Magicmark; Invitrogen Life Technologies) on a NuPAGE 4–12% bis-Tris gel (Invitrogen Life Technologies). The proteins were electroblotted onto a polyvinylidene difluoride membrane (Amersham Biosciences). Unspecific binding was blocked with TBS-tween 3% milk. After blocking, the membranes were incubated with the primary Ab in blocking buffer overnight at 4 °C. The blots were developed using the proper secondary HRP-labeled Ab and visualized with a LAS 1000 camera (Fuji). To confirm sample loading and transfer, membranes were incubated in stripping buffer and re-blocked for 1 h and then reprobed using anti-GAPDH (6C5, Ambion Ltd.). STAT1 P-Tyr701, STAT3 P-Tyr705, STAT4 P-Tyr693, STAT5 P-Tyr694 rabbit antibodies and anti-rabbit HRP labeled antibodies (Cell Signaling), polyclonal goat anti-FOXP3 (Abcam) and anti-goat HRP labeled antibody (Santa Cruz).

2.10 Amplification of FOXP3 promoter fragments

FOXP3 promoter fragments were amplified by conventional PCR using the biotinylated reverse primer 5'-bio-ACCTTACCTGGCTGGAATCACG-3' situated 177 bp downstream of the TSS. Multiple forward primers were designed to generate FOXP3 promoter fragments of different length (Table 2). Reactions were conducted in 75 mM Tris HCl pH 8.8, 20 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.2 mM dNTP's, 0.2 μM of each primer, 6 μg/ml template DNA (FOXP3 promoter in pGL3) and 1.25 U of recombinant Taq DNA polymerase (Fermentas). The same PCR conditions were used for the amplification of all

the products: Initial denaturation step (2 min, 94 °C), 42 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 60 s, final elongation step (7 min, 72 °C). PCR products were purified by ethanol precipitation: 1/10 volume of 3 M sodium acetate and 3 volumes of 100 % ethanol was added to the PCR products, mixed and frozen for 1 h at -80 °C and DNA was collected by a centrifugation step (30 min, maximum speed, 4 °C). The supernatant was removed and residual ethanol was allowed to evaporate. The pellet was eluted in blocking buffer (PBS, 0.1 % BSA, 0.05 % Tween20). Quantification of the FOXP3 promoter probe was done with MassRuler DNA Ladder Mix (Fermentas, Nunningen, Switzerland) on a 1 % agarose gel using AIDA image analyzer software (Raytest).

Primers	Sequence
FOXP3 promoter -1/+177 sense	AAGCCAGGCTGATCCTTTTCTGT
FOXP3 promoter -59/+177 sense	CGTGGTTTTTCTTCTCGGTA
FOXP3 promoter -210/+177 sense	AGTCTCATAATCAAGAAAAGG
FOXP3 promoter -314/+177 sense	TCTCATTGATACCTCTCACCT
FOXP3 promoter -365/+177 sense	AACTCTATACACTTTTGTTTTAAA
FOXP3 promoter -420/+177 sense	CTCGGGTTGGCCCTGTGATT
FOXP3 promoter antisense	ACCTTACCTGGCTGGAATCACG

Table 2 Primer for amplification of FOXP3 promoter constructs

2.11 FOXP3 promoter ELISA

Nuclear extraction was performed as follow. The cells were pelleted and resuspended in buffer C (20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, protease inhibitors (Roche Diagnostics), and 0.1% Nonidet P-40), and lysed on ice for 15 min. Insoluble material was removed by centrifugation. The supernatant was diluted 1/3 with buffer D (as buffer C, but without NaCl). 384 well plates, precoated with streptavidin (Pierce) were washed 3 times with washing buffer (PBS, 0.05 % Tween20). Biotinylated FOXP3 promoter/oligonucleotides probes were added (1 pmol per well; 50 fmol/μl) and incubated for 1 h at RT. After 3 washing steps with washing buffer, nuclear extract was added (concentration > 0.2 μg/μl) and

incubated overnight at 4 °C. The lysates were incubated with 10 μg of poly(deoxyinosinic-deoxycytidylic acid) (Sigma-Aldrich). The plate was washed 3 times with buffer C/D and primary antibody (rabbit anti-STAT1, 1:200 in buffer C/D, Cell Signaling) was added and incubated at 4 °C for 2 h. After 3 washing steps with buffer C/D, secondary antibody (anti-rabbit IgG-HRP, 1:3000 in buffer C/D, Cell Signaling) was added and the plate was incubated for 1 h at 4 °C. The wells were washed 4 times with buffer C/D before adding substrate reagent (R&D system). The colorimetric reaction was stopped after 2 to 10 min by adding 2M H_2SO_4. Absorbance at 450 nm was measured using a conventional microplate reader (Berthold Technologies).

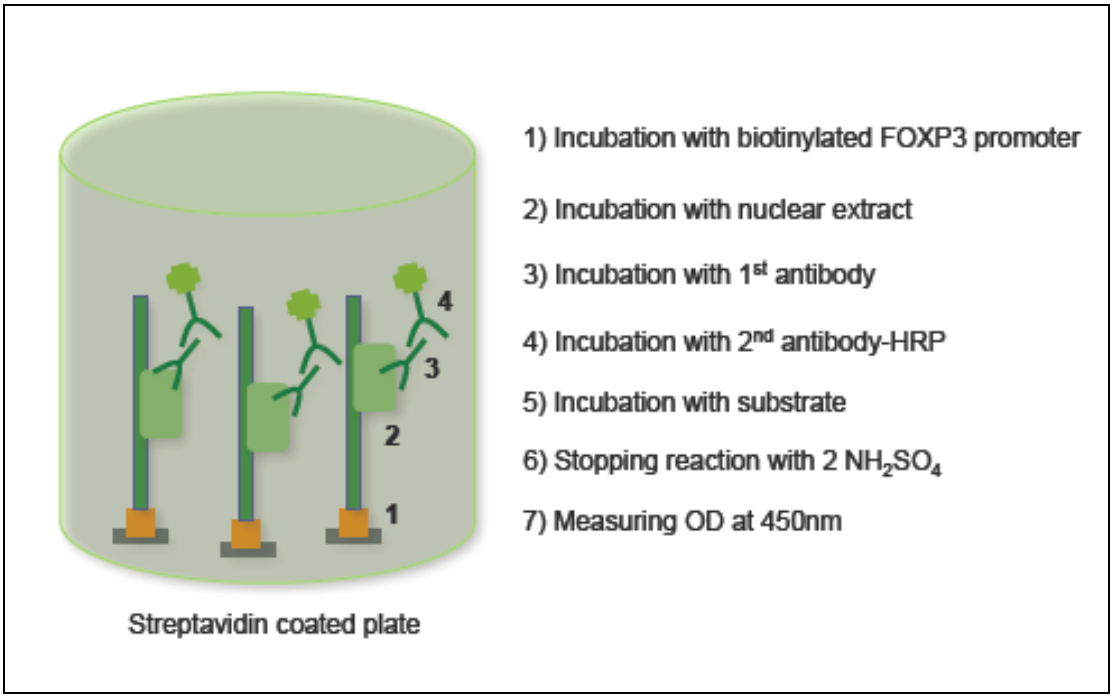

Figure 9: Schema depicting the steps for the Pull Down ELISA

2.12 Pull Down

$CD4^+$ T cells were stimulated with IL-27 (160 ng/ml) and anti-CD3 and anti-CD28 (2.5 μg/ml) during 1 h at 37 °C. Cells were lysed by sonication in HKMG buffer (10 mM Hepes, pH 7.9, 100 mM KCl, 5 mM MgCl2, 10% glycerol, 1 mM DTT, 0.5% Nonidet P-40) containing protease inhibitor cocktail (Roche Diagnostic). Cells lysate was precleared streptavidin-agarose beads for 1 h

(Amersham Biosciences), then incubated with biotinylated double-stranded oligonucleotides (Table 3) and poly (deoxyinosinic-deoxycytidylic acid) (Sigma-Aldrich) for 4 h at 4 °C. DNA-bound proteins were collected with streptavidin-agarose beads for 1 h. The beads were washed 3 times with buffer HKMG and resuspended in NuPAGE loading buffer (Invitrogen Life Technologies), heated to 55 °C for 15 min, and the eluants were separated on a SDS-polyacrylamide gel and identified by Western Blotting.

Oligonucleotides	Sequence
FOXP3 promoter region (-381/-351) WT	GTTTTTTTTTTTTTCAAACTCTATACACTT
FOXP3 promoter region (-381/-351) M	GTTTTTTTTGGTTTCACACTCTATACACTT
FOXP3 promoter region (-361/-331) WT	CTATACACTTTTGTTTTAAAAACTGTGGTT
FOXP3 promoter region (-361/-331) M	CTATACACTTTGGTGTTCAACACTGTGGTT
FOXP3 promoter region (-111/-81) WT	AAAAAATTTGGATTATTAGAAGAGAGAGGT
FOXP3 promoter region (-111/-81) M	AAAAAATTTGGATGATTAGCAGAGAGAGGT
FOXP3 promoter region (-21/+11) WT	TGATACGTGACAGTTTCCCACAAGCCAGGC
FOXP3 promoter region (-21/+11) M	TGATACGTGACAGTTGCCCACAAGCCAGGC
STAT consensus sequence WT	CATGTTATGCATATTCCGAGAAGTG
STAT consensus sequence M	CATGTTATGCATATGCCGACAAGTG

Table 3 Oligonucleotides for pull down analysis

2.13 Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using ChIP assay kit following the recommendations of the supplier (Upstate Biotechnology). For precipitation, 3 μg of Ab against STAT1 (M-22), STAT3 (H-190), STAT4 (H-119), STAT5 (H-134) (Santa Cruz) or anti-acetyl histone H4 (Upstate Biotechnology) were used along with a normal rabbit IgG control (Santa Cruz). Primers addressing the FOXP3 promoter region are listed in Table 4. The conventional PCR products were visualized using an ethidium bromide gel. The real-time PCR data were analyzed in relation to the input DNA using the following expression: $E^{([DNAInput]-[DNAIP])}$.

Primers	Sequence
RT-PCR FOXP3 promoter region (-501/-416) sense	CACATAGAGCTTCAGATTCTCTTTCTTT
RT-PCR FOXP3 promoter region (-501/-416) antisense	CCGAGGCAGGCAGAGACA
RT-PCR FOXP3 promoter region (-204/-114) sense	ATAATCAAGAAAAGGAGAAACACAGAGA
RT-PCR FOXP3 promoter region (-204/-114) antisense	TGAGTGTGTGCGCTGATAATCA
RT-PCR FOXP3 promoter region (-86/+3) sense	AGAGGTCTGCGGCTTCCA
RT-PCR FOXP3 promoter region (-86/+3) antisense	GGAAACTGTCACGTATCAAAAACAA

Table 4 Primers for ChIP analysis

2.14 Transfections and reporter gene assays

T cells were pre-activated in complete RPMI (Life Technologies) containing rhIL-2 (30 ng/ml) and PHA (2 μg/ml) overnight. For the co-transfection, an amount of 3 μg of the pGL3-FOXP3 promoter luciferase reporter vector and 2 μg of the STAT-pCMV-XL4 was added to 6-10 x10^6 $CD4^+$ T cells resuspended in 100 μl of Nucleofector™ solution (Amaxa Biosystems) and electroporated using the T-23 program of the Nucleofector™. For the single transfection, 5 μg of the pGL3-Basic/FOXP3 promoter luciferase reporter vector were used. After a 12 h culture in serum-free conditions and stimulation as indicated in the figures, luciferase activity in cell lysates was measured by the dual luciferase assay system (Promega Biotech) according to the manufacturer's instructions in a Berthold Lumat LB 9507 luminometer.

2.15 Cloning of the FOXP3 promoter, and construction of mutant constructs

The human FOXP3 promoter was cloned into the pGL3 vector (Promega Biotech) to generate the pGL3 FOXP3 -511/+177 as previously described [125]. Site-directed mutagenesis in the FOXP3 promoter region was introduced using the QuickChange kit (Stratagene), according to the manufacturer's instructions. The constructs were generated by using pGL3 -511/+177 as template. Primers used to generate the individual constructs are listed in Table 5.

Primers	Sequences
FOXP3 promoter site -99 sense	GAAAAAAATTTGGATTATTAGCAGAGAGAGGTCTGCGGCTTC
FOXP3 promoter site -99 antisense	GAAGCCGCAGACCTCTCTCTGCTAATAATCCAAATTTTTTTC
FOXP3 promoter site -7 sense	GATACGTGACAGTGTCCCACAAGCCAG
FOXP3 promoter site -7 antisense	CTGGCTTGTGGGACACTGTCACGTATC
FOXP3 promoter site UR sense	TTGTTTTAAAAACTGTGGTTTCTCGAGAGCCCTATTATCTCATTGATACC
FOXP3 promoter site UR antisense	GGTATCAATGAGATAATAGGGCTCTCGAGAAACCACAGTTTTTAAAACAA

Table 5 Primers for Mutagenesis

2.16 Statistical analysis

The non-parametric Wilcoxon matched paired test and linear regression were used to test statistical significance using the GraphPad Prism software (GraphPad Software). P-values ≤0.05 were considered as significant.

Results

2.1 The FOXP3 expression is regulated by Th1 cytokines

The WSX-I/TCCR subunit, which is exclusive to the IL-27 receptor, is expressed on CD4$^+$ naïve T cells (Figure 10A) as well as on natural Treg cells [157]. Its high expression on *in vitro* differentiated iTreg cells (Figure 10B) suggests that IL-27, which can directly act on naïve cells, regulates Treg cells differentiation or function.

To elucidate whether IL-27 has a role during iTreg cell differentiation, we cultured human naïve CD4$^+$ T cells under conditions leading to the differentiation of iTreg cells (with TGF-β) ± IL-27. Even though TGF-β stimulation resulted in elevated FOXP3 mRNA after 3 days (54.1±11.8 fold) and protein after 5 days (33.4±6.4%), IL-27 further significantly increased this induction (mRNA=98.2±19.9 fold, protein=44.1±6.5%, p=0.0244) (Figure 11). To determine whether IL-27 was the only Th1-inducing cytokine influencing the FOXP3 expression in the presence of TGF-β, we also cultured naïve CD4$^+$ T cells under iTreg condition in the presence of IL-12 or IFN-γ. TGF-β-induced FOXP3 expression level was maintained in the presence of IL-12 (mRNA=56.1±28.9 fold and protein=29.6±6.7%), whereas IFN-γ acted like IL-27, as an amplifier of TGF-β-induced response (mRNA=97.3±36.7 fold and protein=47.7±8.2%, p=0.0312) (Figure 11).

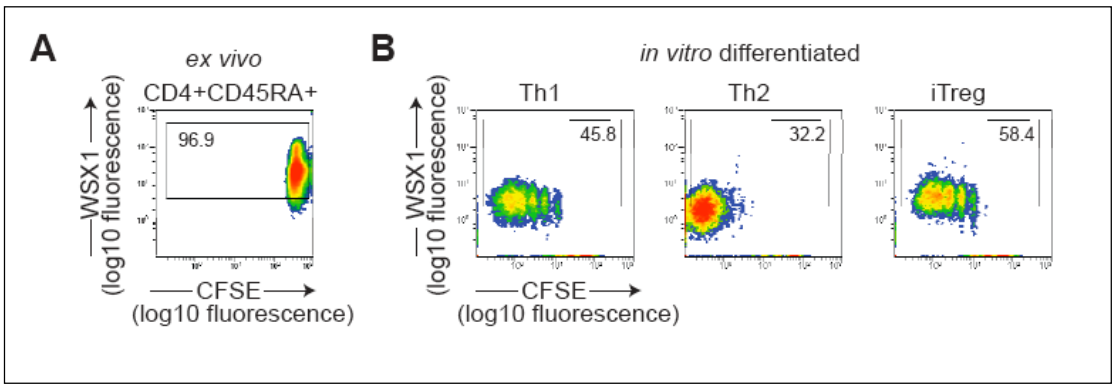

Figure 10: The WSX-1/TCCR subunit is highly expressed on iTreg cells.

FACS analysis of WSX-1/TCCR expression on CFSE labeled A) *ex vivo* CD4$^+$CD45RA$^+$ T cells, and B) differentiated CD4$^+$CD45RA$^+$ T cells during 5 days in Th1, Th2 or iTreg condition.

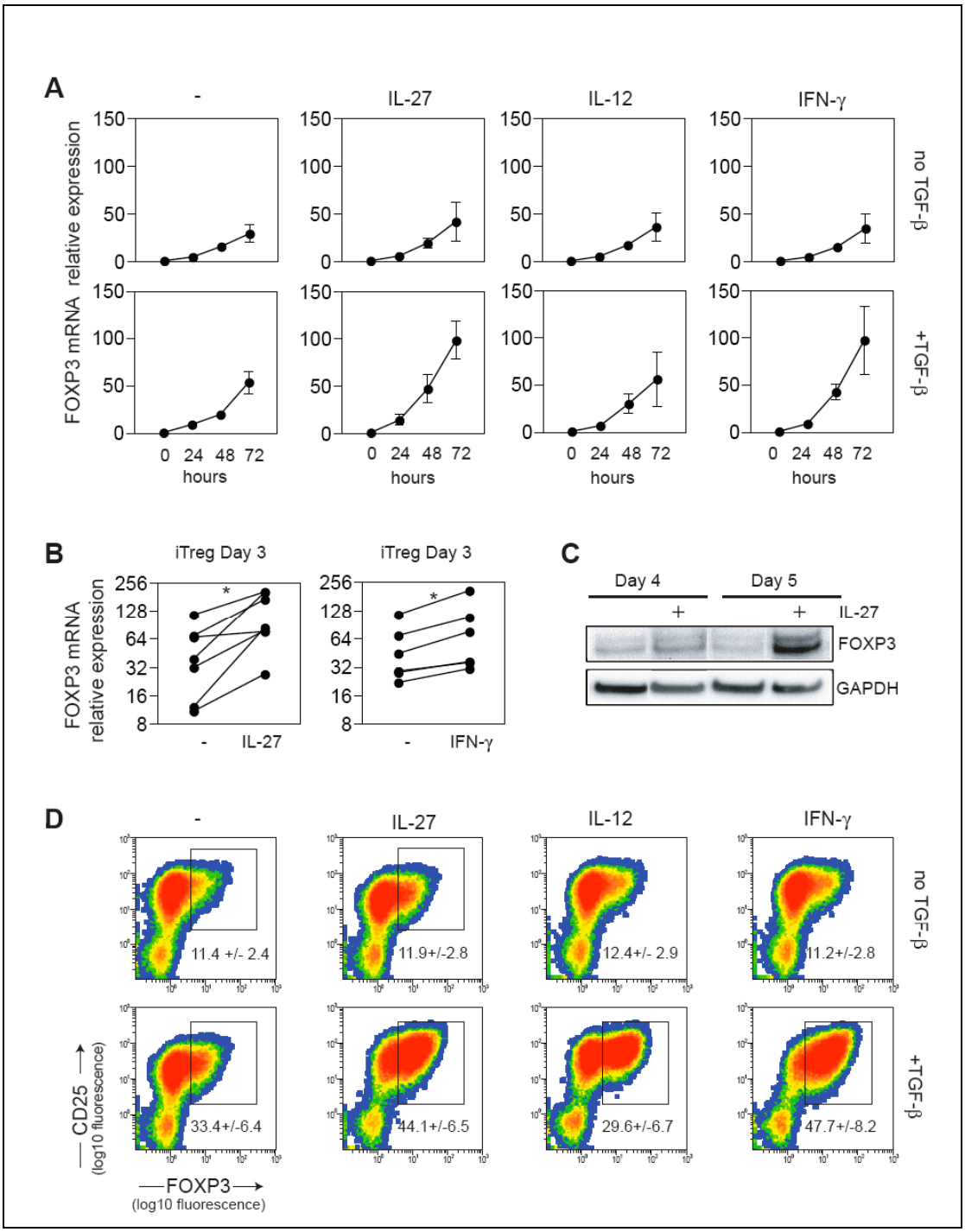

Figure 11: IL-27 and IFN-γ increase TGF-β-induced FOXP3 expression

Human $CD4^+CD45RA^+$ T cells were activated with soluble anti-CD3/CD28 and differentiated in neutral or iTreg driven condition ± IL-27, IL-12 or IFN-γ as indicated. Cells were harvested and FOXP3 expression was analyzed by real-time PCR after A) 24 to 72 h or only B) 3 days and C) by FACS analysis, together with CD25 expression, after 5 days. Symbols represent A) the mean ± s.e.m. of 6-8 independent experiments, or B) different donors. * = $p < 0.05$.

2.2 The cytokine profile of iTreg cells is modified by Th1 cytokines

To further characterize the cell population developing in the presence of Th1-inducing cytokines and TGF-β, we performed IFN-γ intracellular staining (Figure ***12***). Consistent with previous findings [3,158], the frequency of IFN-γ-producing cells increased upon treatment with IL-27, IL-12 or IFN-γ (23.4±5.1 vs 36.6±10.2, 56.1±4.7 and 40.3±9.8 % respectively). IFN-γ production was low in the presence of TGF-β (11.5±4.5%), whereas IL-12-induced IFN-γ production was sustained (49.9±12.4%). The analysis of the supernatants of the above cell cultures by Bio-Plex cytokine bead assay confirmed high amount of IFN-γ production upon culture with IL-12 and TGF-β (Figure 13). TGF-β-mediated suppression of the IFN-γ production was unchanged by IFN-γ or IL-27 and IL-4, IL-10 and IL-17 were merely detectable under all conditions (Figure 13). Taken together, those findings are ruling out a differentiation into Th1, Th2 or Th17 cells.

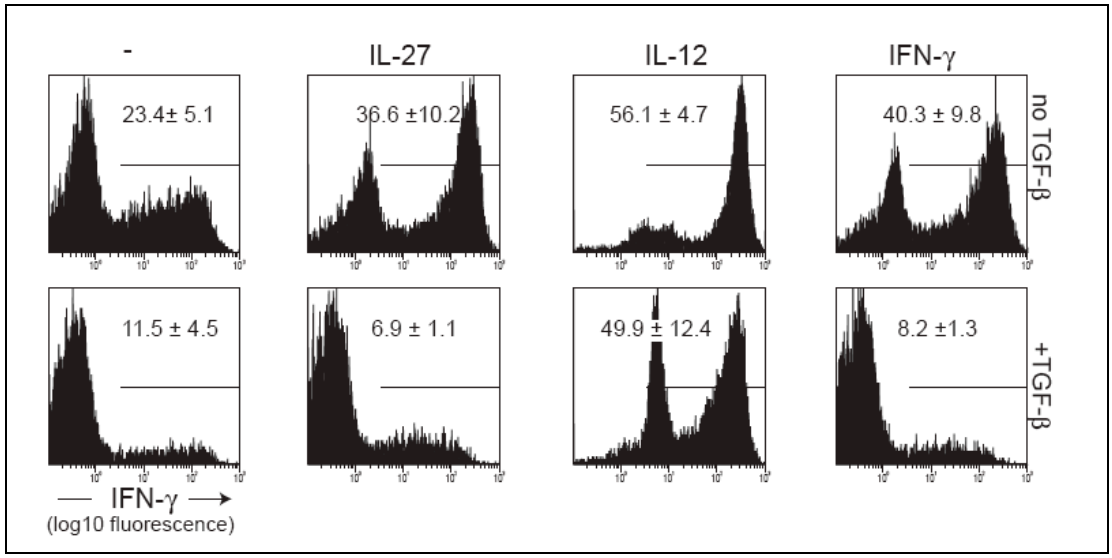

Figure 12: TGF-β impairs IFN-γ inducing capcity of IL-27 and IFN-γ

Human $CD4^+CD45RA^+$ T cells were activated with soluble anti-CD3/CD28 and differentiated in neutral or iTreg driven condition ± IL-27, IL-12 or IFN-γ as indicated. After 12 days, intracellular IFN-γ expression was analyzed by FACS following PMA/ionomycin stimulation. FACS data are representative of 5 independent experiments.

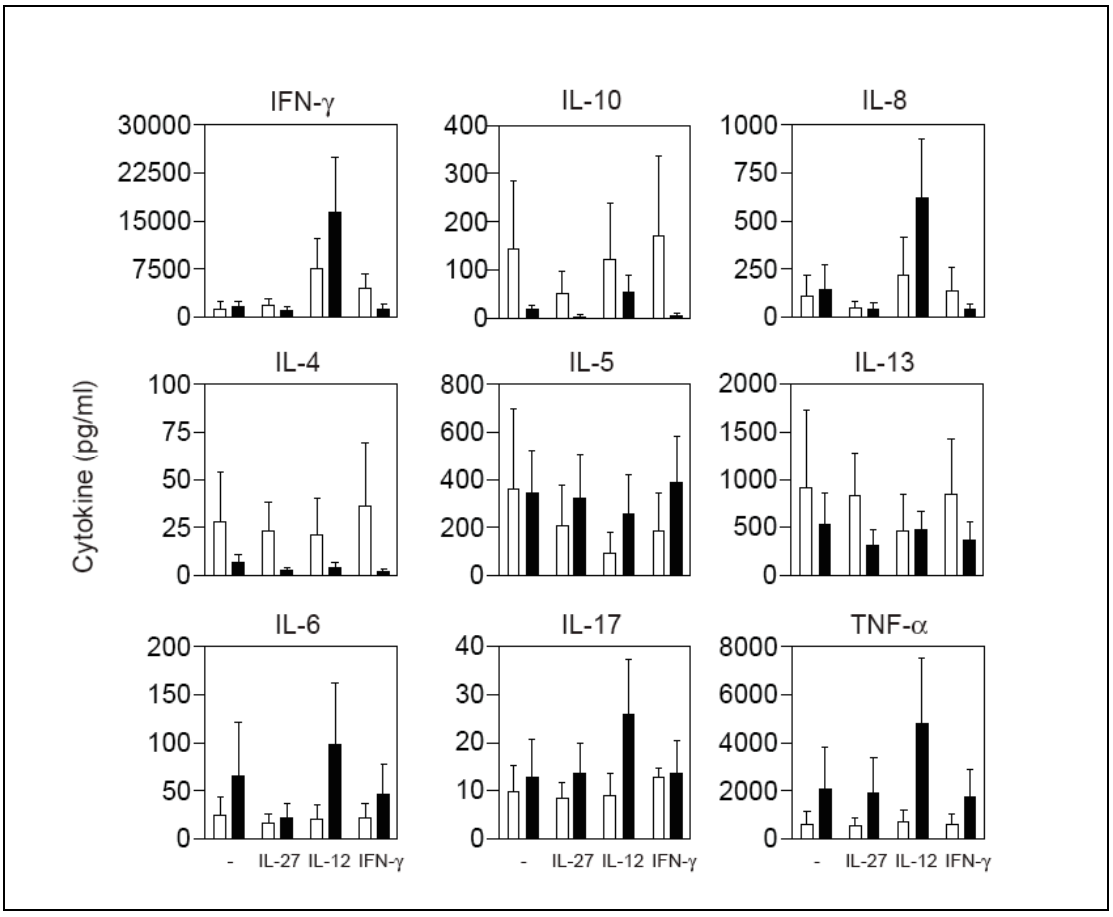

Figure 13: The cytokine profile of iTreg cells in the presence of Th1 cytokines

Human $CD4^+CD45RA^+$ T cells were activated with soluble anti-CD3/CD28 and differentiated in neutral (white bars) or iTreg (black bars) driven condition ± IL-27, IL-12 or IFN-γ as indicated. After 12 days, cells were restimulated for 48 h with anti-CD3/CD28 and cytokine expression was analyzed by Bio-Plex beads assay. Bars show the mean ± s.e.m. of 3 independent experiments.

2.3 The suppressive capacity of iTreg is sustained with Th1 cytokines

To investigate whether the increased expression of FOXP3 resulted in the acquisition of suppressive capacity, we tested the *in vitro* differentiated iTreg cells for their capability to suppress the proliferation of autologous $CD4^+$ responder T cells. All *in vitro* differentiated iTreg cells exhibited suppressive ability in a dilution-dependent manner (Figure 14). In contrast to natural Treg cells that are anergic, iTreg cells possess the capacity to proliferate upon stimulation. To investigate the effect of this proliferation in suppression assays, we included stimulated total $CD4^+$ T cells as a control (Figure 14B). These cells were stimulated with anti-CD3, anti-CD28 and IL-2 in parallel to the *in vitro* differentiation of iTreg, and used as suppressors in the suppression assay. The iTreg-mediated suppression of the proliferation of $CD4^+$ responder T cells was higher than the one observed with stimulated total $CD4^+$ T cells. Taken together, these findings suggest the induction of functional iTreg cells in the presence of TGF-β and IL-27 or IFN-γ.

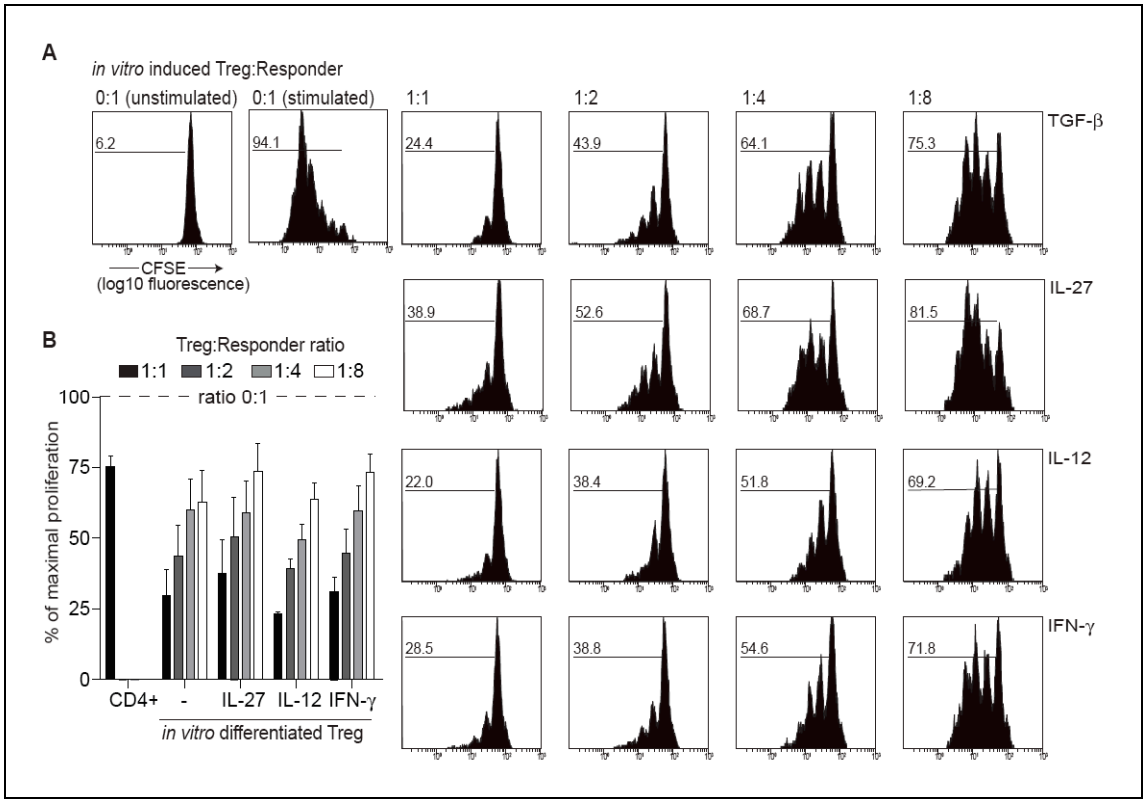

Figure 14: Suppressive capacity of iTreg cells cultured with Th1-inducing cytokines.

Human $CD4^+CD45RA^+$ T cells were *in vitro* differentiated towards iTreg cells ± IL-27, IL-12 or IFN-γ. After 10 days of culture, iTreg cells were activated with soluble anti-CD3, autologous irradiated PBMC and CFSE-labeled $CD4^+$ responder T cells in different ratios as indicated. The proliferation of $CD4^+$ responder T cells is depicted in A). Data are representative of 4 independent experiments. B) Percentage of the maximal proliferation. Stimulated $CD4^+$ responder T cells without iTreg is set as 100%. Bars show the mean ± s.e.m. of 4 independent experiments.

Recently, the EBI3-IL-12p35 heterodimeric complex, designated as IL-35, was shown to be constitutively produced by mouse Treg cells [79] and to be required for their suppressive activity. To confirm the regulatory phenotype of our iTreg cells, we analyzed the expression of both IL-35 subunits (Figure 15). IL-12p35 and EBI3 were both detectable in *in vitro* differentiated iTreg at higher level than in *ex vivo* $CD4^+$ naïve T cells (Day 0).

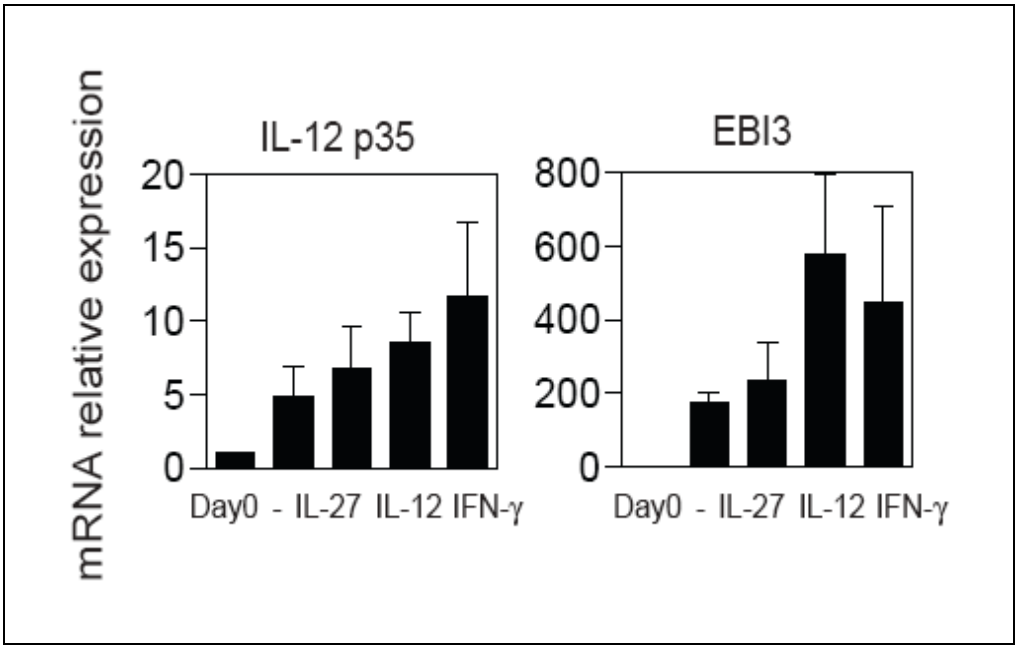

Figure 15: iTreg cells can produce IL-35

Human $CD4^+CD45RA^+$ T cells were *in vitro* differentiated towards iTreg cells ± IL-27, IL-12 or IFN-γ. Quantitative RT-PCR of IL-12 p35 and EBI3 of iTreg cells 10 days post differentiation. Bars show the mean ± s.e.m. of 3 independent experiments.

To further investigate the phenotype of the cells cultured in the presence of IL-27 and TGF-β, we assessed the expression of distinctive Treg markers [86-88,159]. Because *in vitro* activated cells express high levels of CD25 independently of their subset, CD25 expression was not assessed. The comparable expression level of CD103, CTLA-4 (CD152), PD-1 and GITR, following iTreg or iTreg+IL-27 cell differentiation confirmed the regulatory phenotype of Th1 cytokines treated iTreg cells (Figure 16). Taken together, these findings suggest an induction of functional iTreg cells in the presence of TGF-β and IFN-γ or IL-27.

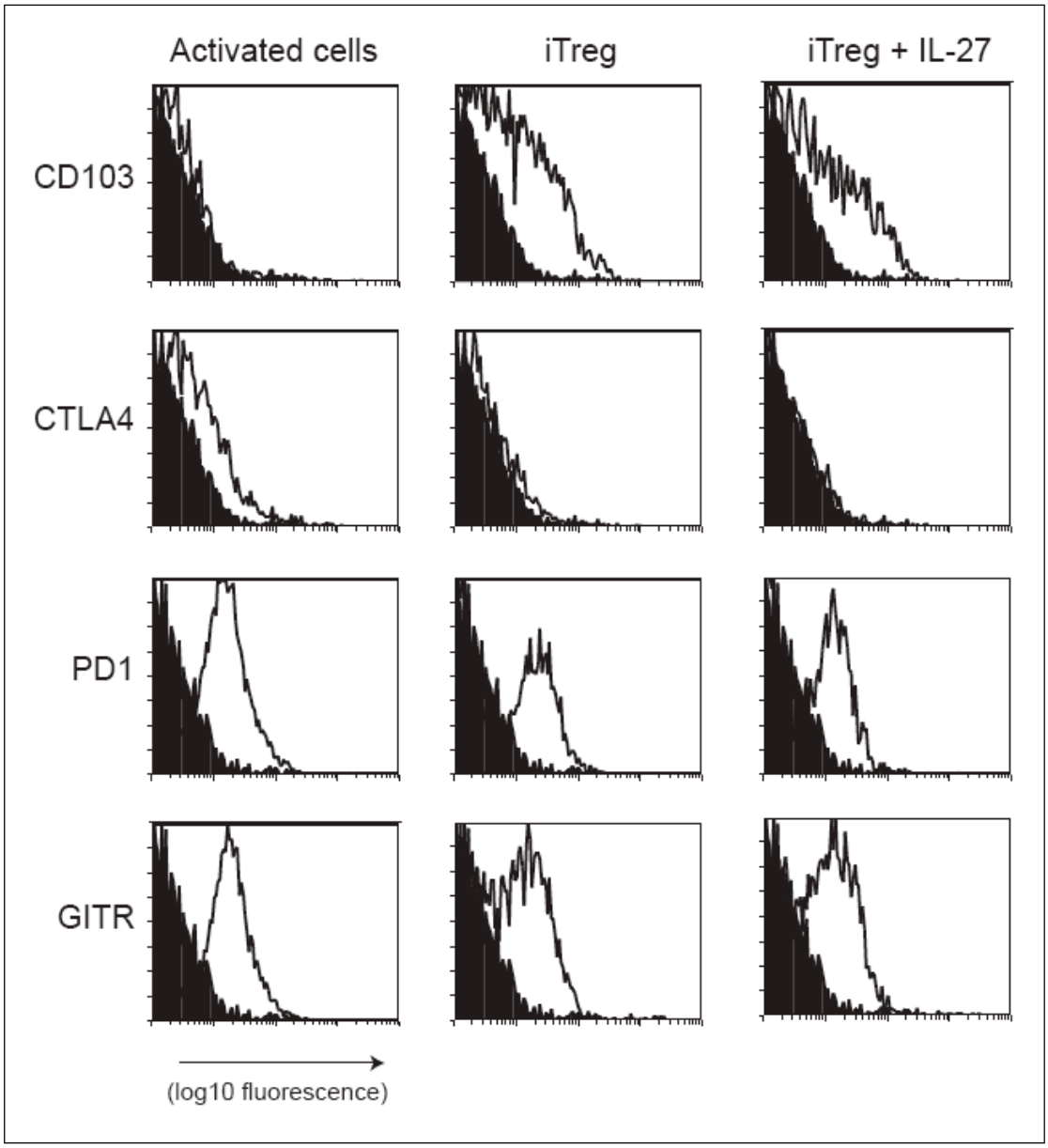

Figure 16: Treg markers characterization

Human $CD4^{+}CD45RA^{+}$ T cells were activated with anti-CD3/28 or *in vitro* differentiated towards iTreg cells ± IL-27. FACS analysis of distinctive Treg markers were performed after 7 days post differentiation. Data are representative of 4 independent experiments.

2.3 The indirect influence of IFN-γ

To further investigate the contribution of IFN-γ and IL-27 on the induction of FOXP3, we titrated both cytokines and analyzed FOXP3 expression. While a concentration-dependent effect was observed for IL-27, IFN-γ did not modify the percentage of FOXP3 positive cells at any concentration (Figure 17A-B). To further examine the role of IFN-γ, we stimulated naïve $CD4^+$ T cells ± anti-IFN-γ. FOXP3 mRNA and protein levels were comparable with or without blocking the effect of IFN-γ (Figure 17C-D).

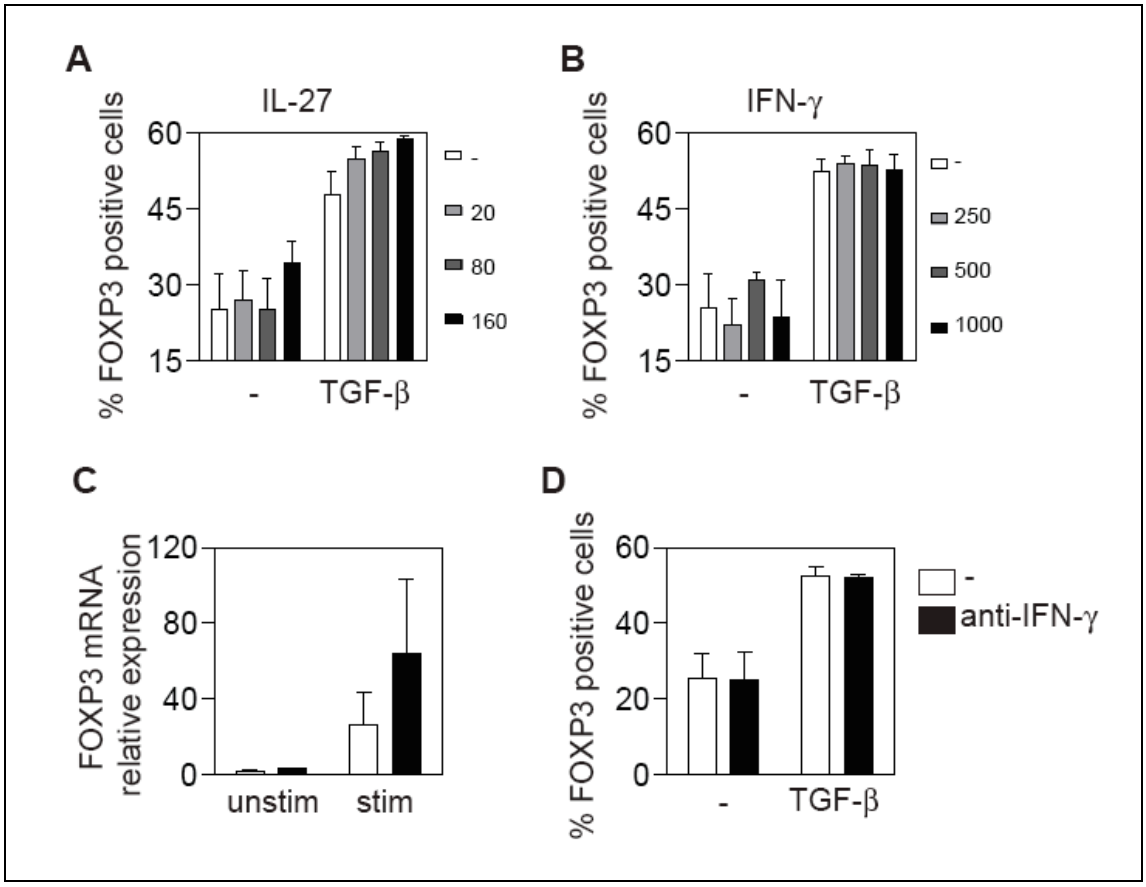

Figure 17: The influence of IFN-γ on FOXP3 expression is indirect

Human $CD4^+CD45RA^+$ T cells were *in vitro* differentiated with or without TGF-β ± increasing concentration of A) IL-27 (0, 20, 80 and 160 ng/ml) and B) IFN-γ.(0, 250, 500 and 1000 U/ml) and analysed by FACS for FOXP3 positive cells.
Human $CD4^+CD45RA^+$ T cells were unstimulated or TGF-β stimulated ± anti-IFN-γ and FOXP3 expression was analyzed C) by real time PCR after 3 days and D) by FACS after 5 days. Bars show the mean ± s.e.m. of 3 independent experiments.

Recently, it was demonstrated that IFN-γ-induced STAT1 binds to the IL-27 promoter and induces IL-27 expression [145]. IL-27, which has been shown to be produced by dendritics cells, monocytes and endothelials cells, is also expressed by the T cell immortal Jurkat cell line. Both subunits of IL-27 were detectable after 48 hours stimulation in the presence of IFN-γ (Figure 18). Taken together, these findings suggest that IFN-γ indirectly influences FOXP3 expression via the production of IL-27 in human T cells.

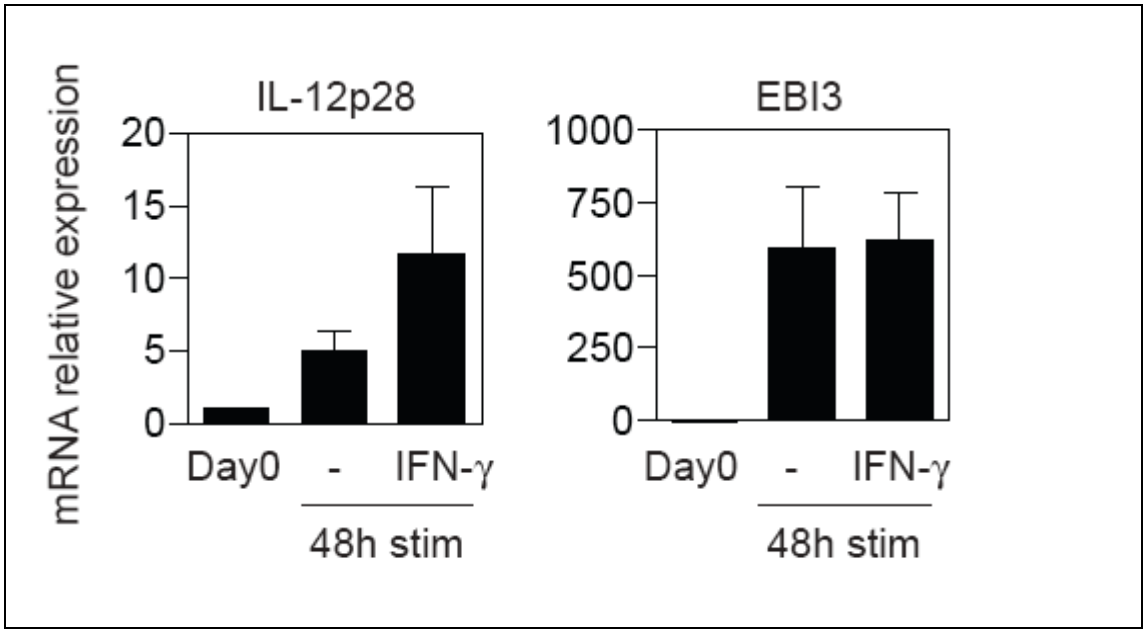

Figure 18: IL-27 is induced by IFN-γ

Quantitative RT-PCR of IL-12 p28 and EBI3 of human $CD4^+CD45RA^+$ T cells before and after 48h of stimulation with IFN-γ. Bars show the mean ± s.e.m. of 3 independent experiments.

2.4 T-bet influences the FOXP3 expression

To investigate the role of the Th1-specific transcription factor T-bet on the expression of FOXP3, T-bet expression during the human naïve $CD4^+$ T cell differentiation with or without TGF-β ± IL-27, IL-12 or IFN-γ was analyzed. In accordance with their role as Th1-inducing cytokines, IL-27, IL-12 and IFN-γ induced T-bet mRNA expression in the absence of TGF-β. Even though TGF-β stimulation resulted in reduced T-bet mRNA levels, Th1 cytokines remained capable of inducing T-bet mRNA. Since T-bet was still detectable under iTreg culture conditions, we investigated whether its expression correlated with FOXP3 expression. In harmony with the potentiation of FOXP3 by Th1 cytokines, T-bet expression positively correlated with FOXP3 expression (r^2= 0.393, p=0.0004) (Figure 20). However, the weak correlation observed suggest the implication of complementary and more direct mechanisms in the induction of FOXP3.

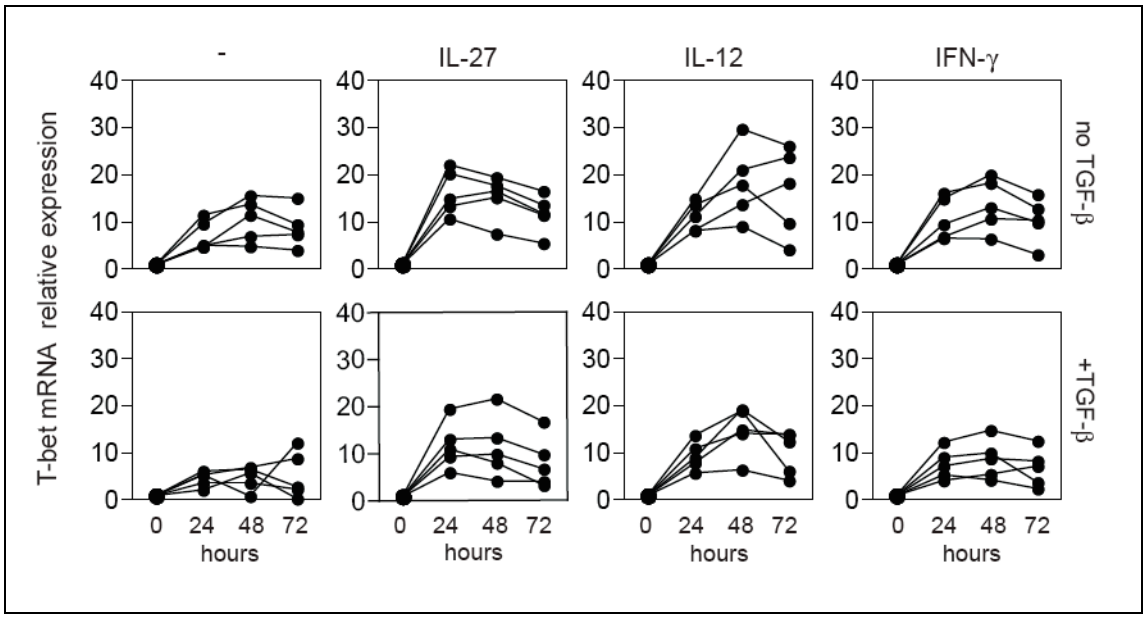

Figure 19: T-bet is expressed during iTreg cell differentiation

Human $CD4^+CD45RA^+$ T cells were activated with soluble anti-CD3/CD28 and differentiated in iTreg driven condition ± IL-27, IL-12 or IFN-g as indicated. Cells were harvested and FOXP3 expression was analyzed by real-time PCR after 24 to 72 h. Symbols represent different donors.

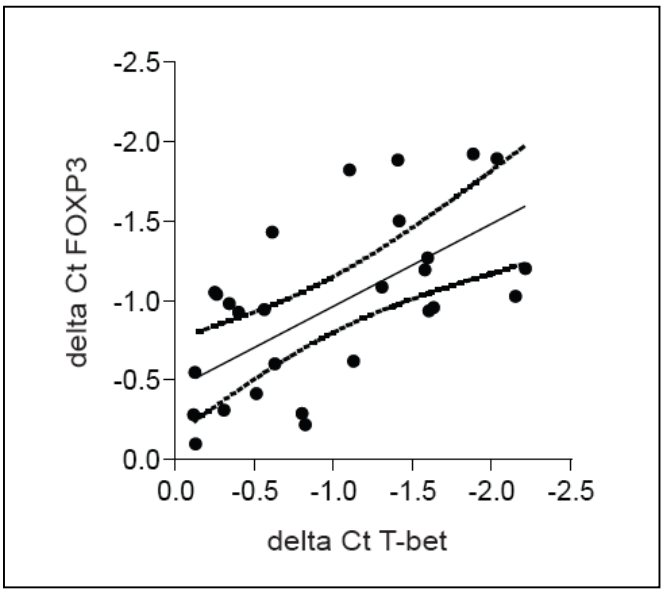

Figure 20: T-bet expression correlates with FOXP3 expression.

The real time PCR Ct value of FOXP3 and T-bet were substracted from the Ct value of the house keeping gene Elongation factor 1. A linear regression was performed. Straight line represent linear regression. Dashed lines represent 95% confident intervals. Symbols represent different donors.

2.5 The FOXP3 promoter contains STAT binding sites

Several studies reported a potential role of STAT molecules on the foxp3 gene regulation [153]. STAT3 and STAT5 binding to FOXP3 promoter, to the untranslated region (UTR) and to exons of the foxp3 gene have been reported [126,138,152]. TESS analysis of the human FOXP3 promoter predicted 4 putative STAT binding sites at 373bp, 351bp, 99bp and 7bp upstream of the Transcription Start Site (TSS)). Positions of STAT binding sites within the FOXP3 promoter/gene are summarized (

Figure ***21***).

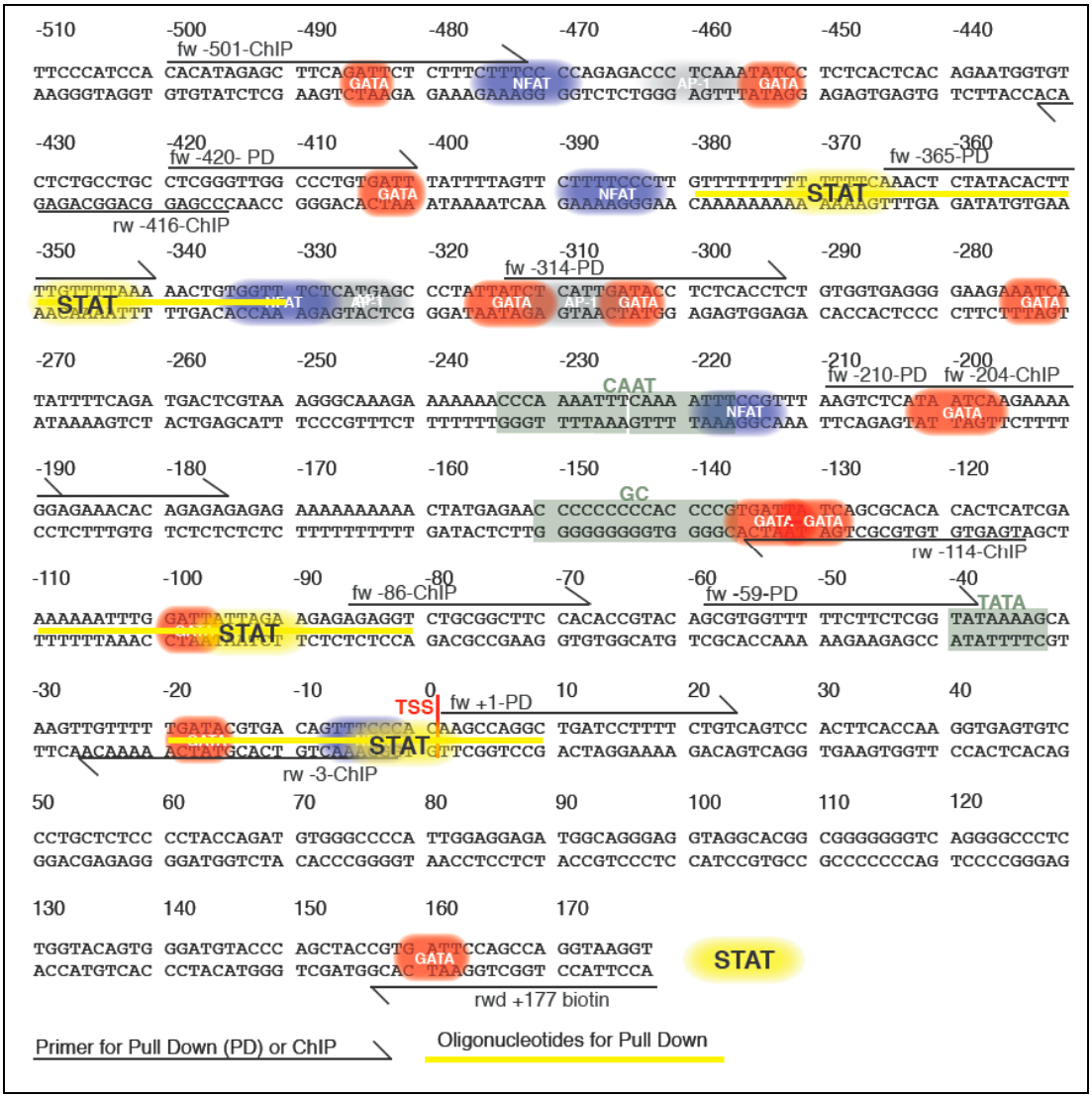

Figure 21: FOXP3 promoter sequence
Sequence depicting the STAT binding sites and the position of primers and oligonucleotides used for the Pull Down and the ChIP analysis.

Both IL-27 and IFN-γ are known to induce STAT1 Tyr701 phosphorylation and activation. Since the induction of FOXP3 is dependent on TGF-β signalling, we analyzed whether IL-27 and IFN-γ-induced STAT1 phosphorylation occurred in the presence of TGF-β. Both cytokines induced STAT1 phosphorylation, which enable its DNA binding, whereas IL-12 unaffected this molecule (Figure 22).

Direct binding of STAT1 to the FOXP3 promoter was investigated in a FOXP3 promoter ELISA analysis. Oligonucleotides corresponding to different lengths of the FOXP3 promoter were coated on ELISA plates. STAT1 binding was detectable by chemiluminescence following incubation with nuclear extract from IL-27-stimulated $CD4^+$ T cells and suitable antibodies and detection reagents. STAT1 strongly bound to its specific consensus sequence, but not to the mutated sequence, which validated the specificity of the assay. STAT1 bound the full-length FOXP3 promoter construct (-420/+177) containing all STAT binding sites. A similar binding was observed in FOXP3 promoter constructs lacking sites at positions -373bp and/or -351bp (-365/+177, -314/+177, -210/+177), was reduced in the construct lacking the site at position -99bp (-59/+177) and was totally abolished in the construct lacking the site at position -7bp, (-1/+177).

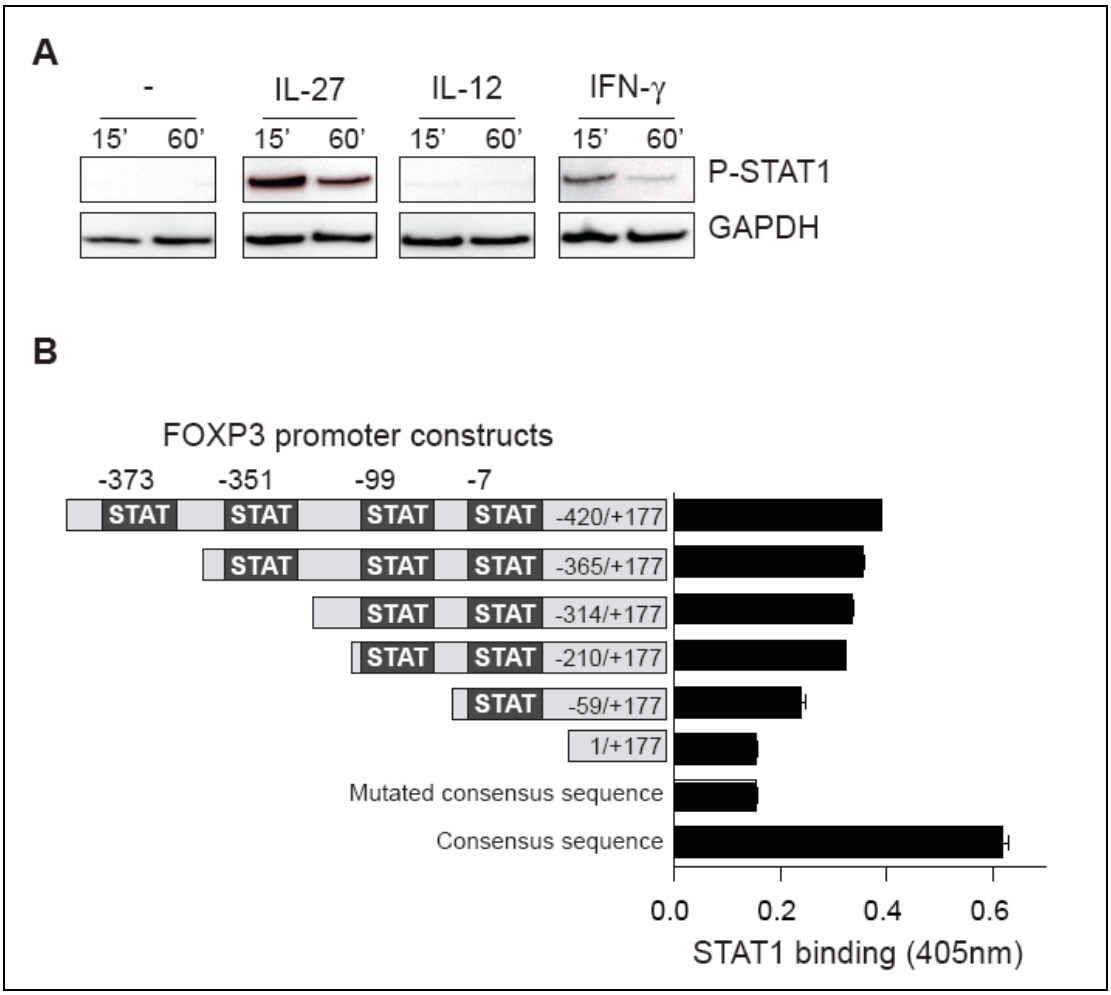

Figure 22: Activated STAT1 can bind to FOXP3 promoter

Primary human $CD4^+$ T cells were stimulated with anti-CD3/28, IL-2, TGF-β ± IL-27, IL-12 or IFN-γ during 15 and 60 min. The nuclear extract was analyzed by Western blot for STAT1 Tyr 701 phosphorylation A). GAPDH was used as an internal loading control. Data are representatives of 3 independent experiments. Primary human $CD4^+$ T cells were stimulated with anti-CD3/28, IL-2, TGF-β and IL-27 for 30 min and the nuclear extract was tested B) for the binding of STAT1 to the consensus sequence or the mutated consensus sequence, and to different lengths of the FOXP3 promoter. Bars show the mean ± S.D. of triplicates representative of 2 independent experiments.

In agreement with the FOXP3 promoter ELISA results (Figure 22), STAT1 binding was observed to the sites -99bp and -7bp in pull down analysis using 30 bp probes containing putative STAT binding sites and the surrounding regions in the FOXP3 promoter (Figure 23). This suggests that activated STAT1 can recognize and bind 2 sequences within the FOXP3 promoter. Because IL-27 can also activate other STAT family members, we further tested the possibility that other IL-27 activated STAT molecules (e.g. STAT3, STAT4 and STAT5) bind the putative STAT binding sites within the FOXP3 promoter. A slight binding of STAT3 was also observed to the site at position -7 and even though STAT4 and STAT5 were activated and able to bind the STAT consensus sequence following IL-27 stimulation, there was no apparent binding to any of the probes (Figure 23 and data not shown).

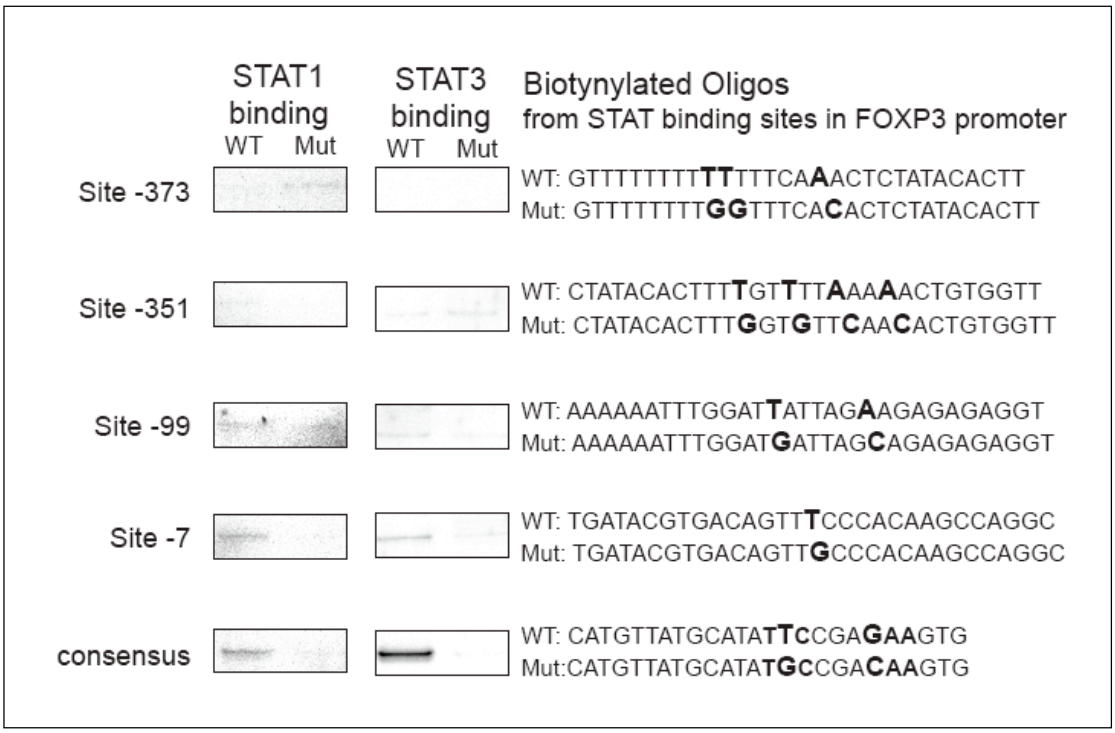

Figure 23: : STAT1 binds to the FOXP3 promoter

Primary human $CD4^+$ T cells were stimulated with anti-CD3/28, IL-2, TGF-β and IL-27 for 30 min and the nuclear extract was tested for the binding of STAT1 and STAT3 to the consensus sequence or the mutated consensus sequence, and to different lengths of the FOXP3 promoter. Data are representative of 3 independent experiments.

2.6 STAT1 binds the FOXP3 proximal promoter in vivo

To clarify the importance of the STAT molecules' binding in regulating *foxp3* gene expression *in vivo*, we performed a ChIP assay. In primary $CD4^+$ naïve T cells, only STAT1 binding was clearly detectable to the FOXP3 promoter following stimulation with TGF-β, anti-CD3, anti-CD28, IL-2 and IL-27 for 30 min (Figure 24). The location of STAT1 binding *in vivo* was evaluated by ChIP assay using PCR primers amplifying 3 regions of the FOXP3 promoter (-501/-416, -204/-114, -86/-3). Coherent with the pull-down analysis (Figure 23), STAT1 was binding to the regions in proximity to the sites at positions -99bp and -7bp (-214/-114, -83/-3) (Figure 24). Thus, STAT1 was the only STAT member binding to sites at positions -99bp and -7bp of the FOXP3 proximal promoter to the chromatin following IL-27 stimulation.

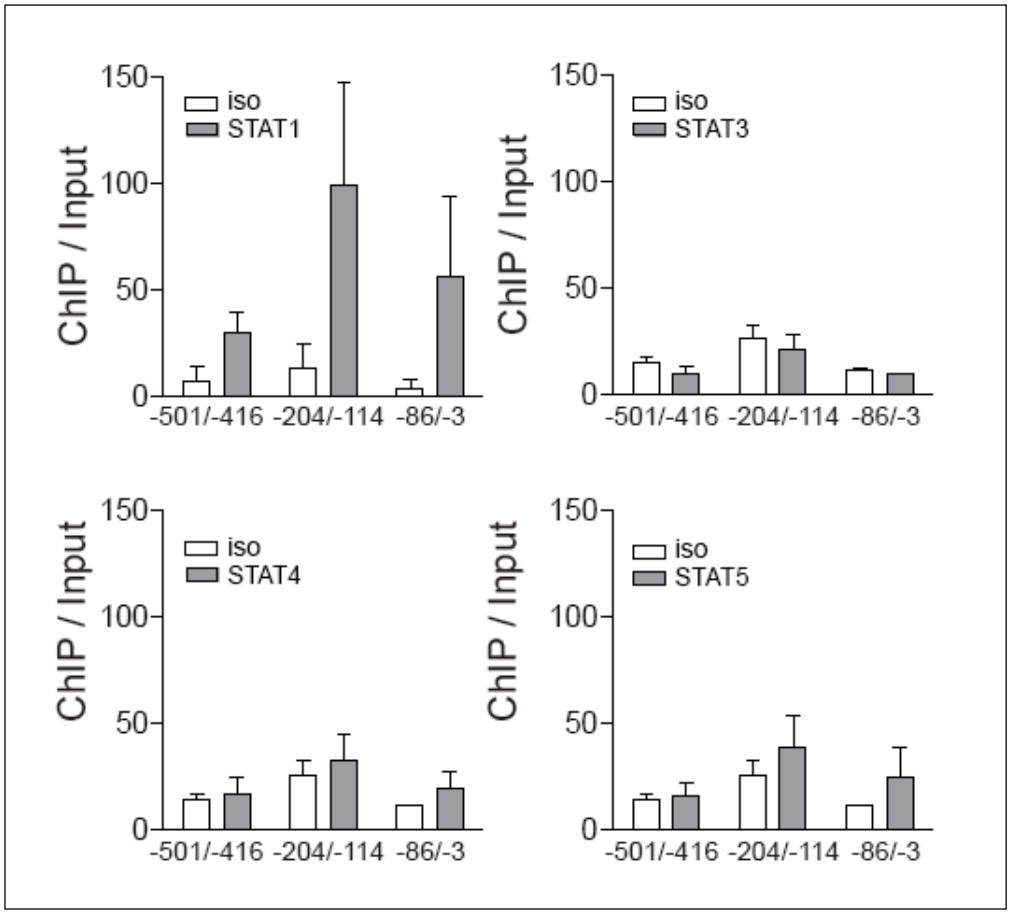

Figure 24: STAT1 is the only IL-27-induced STAT member binding the FOXP3 promoter in vivo

Primary human $CD4^+$ T cells were stimulated with anti-CD3/28, IL-2, TGF-β and IL-27 for 30 min. Cells were analyzed by ChIP for STAT1, STAT3, STAT4 and STAT5 binding to the FOXP3 promoter. The chromatin DNA obtained before (Input) and after immunoprecipitation with anti-STAT antibody or with the respective isotype control was analyzed by real time PCR with 3 specific primers pairs for the FOXP3 promoter. Bars show mean ± s.e.m. of 3 independent experiments.

2.7 IL-27-induced STAT1 regulates foxp3 gene expression

Functional significance of STAT1 binding to the FOXP3 promoter was analyzed by FOXP3 promoter luciferase assay using IL-27-stimulated $CD4^+$ T cells. The strongest luciferase activity was generated with -511/+177 pGL3 FOXP3 promoter reporter plasmid after co-transfection with STAT1 ($p=0.005$) (Figure 25A), reflecting a role of STAT1 in regulating the FOXP3 promoter activity. The respective contribution of the -99bp and -7bp sites in the STAT-mediated regulation of FOXP3 was examined using mutant constructs. The mutations A-92C and T-6G, which abolished the STAT binding capacity, greatly decreased the overall activity of the promoter, whereas an unrelated mutation (AT-327GA) did not change it (Figure 25B). This underlined the importance of these binding sequences in the regulation on the promoter activity. IL-27 slightly increased the transactivation of the FOXP3 promoter luciferase reporter construct ($p=0.0321$) (Figure 25C). This effect was no longer significant in the mutant constructs, suggesting that IL-27 mediated effect takes place through STAT1 binding to the sites at positions -99bp and -7bp.

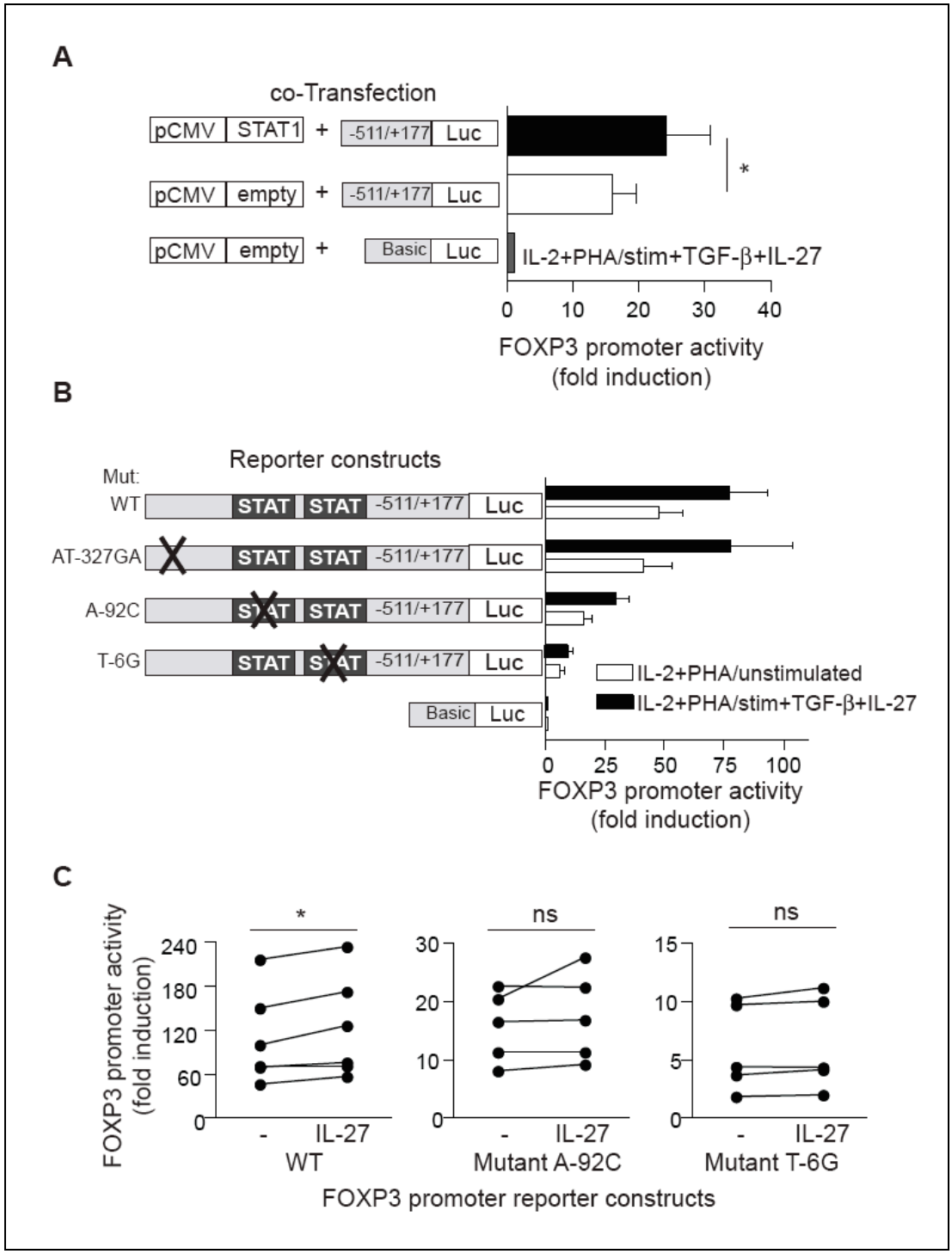

Figure 25: IL-27-induced STAT1 regulates FOXP3 promoter's activity

Primary CD4+ human T cells were activated with IL-2 and PHA. A) Cells were transfected with pGL3 (Basic or FOXP3 -511/+177) and pCMV (empty or STAT1) vectors as indicated and stimulated with anti-CD3/28, IL-2, TGF-β and IL-27 18 h prior measurement. Cells were transfected an empty vector (pGL3 Basic), a WT FOXP3 promoter containing vector or a mutated FOXP3 promoter containing vector and stimulated with B) anti-CD3/28, IL-2, TGF-β and IL-27 or C) anti-CD3/28, IL-2, TGF-β ± IL-27 during 18 h prior measurement for the relative luciferase light units. Unstimulated basic vector is set as 1. Bars show mean ± s.e.m. of 4 to 8 independent experiments. * = $p < 0.05$.

2.8 IL-27 controls foxp3 gene expression at the epigenetic level

STAT1 has been shown to be implicated in chromatin remodeling following IFN-γ stimulation [160]. A single point mutation that prevented its phosphorylation was sufficient to abolish the chromatin remodeling. The role of IL-27 in histone modification of the FOXP3 promoter was investigated by performing ChIP assays with anti-acetyl histone H4 (Figure 27). In iTreg cells (cultured for 8 days) histone H4 molecule was acetylated in the -501/-416 region of the FOXP3 promoter (Figure 27). Addition of IL-27 in the culture induced histone H4 acetylation in the regions near STAT1-binding sequences (-204/-144, -86/-3). This indicated a role of IL-27-induced STAT1 in regulating the FOXP3 proximal promoter by chromatin remodeling.

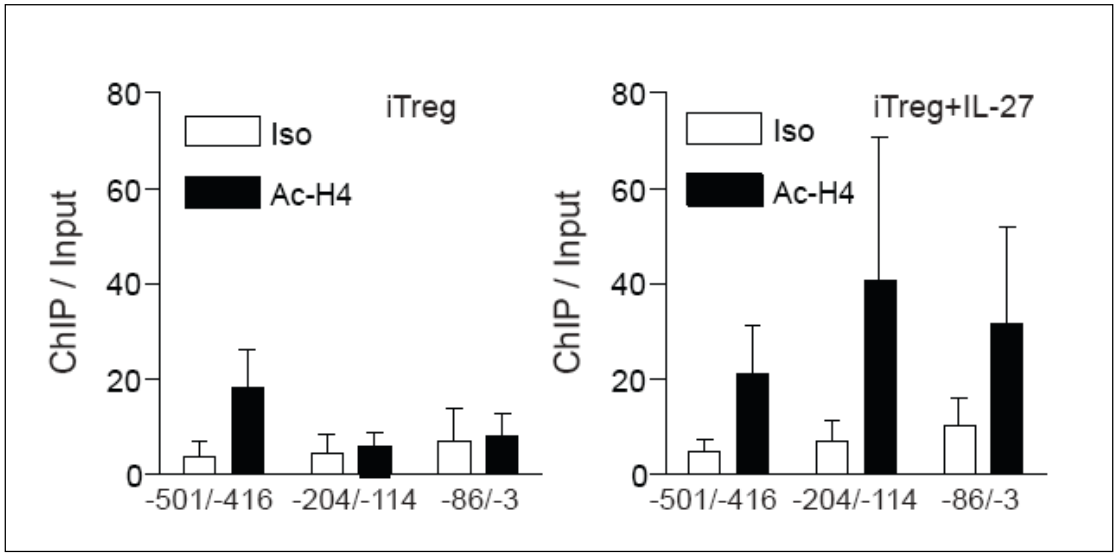

Figure 27: IL-27-induced STAT1 regulates histone acetylation

Human $CD4^+CD45RA^+$ T cells were activated with soluble anti-CD3/CD28 and differentiated in iTreg driven condition without or with IL-27. After 8 days, cells were analyzed by ChIP for histone H4 acetylation in the FOXP3 promoter. The chromatin DNA obtained before (Input) and after immunoprecipitation with anti-acetyl H4 antibody or with the isotype control was analyzed by real-time PCR with 3 specific primers pairs for the FOXP3 promoter. Bars show mean ± s.e.m. of 4 independent experiments.

Collectively, these findings suggest that IL-27 has the capability to act on naïve $CD4^+$ T cells during their differentiation into effector cells. When IL-27 stimulation is coupled with TGF-β stimulation, cells undergo iTreg differentiation with reduced IFN-γ production and potently suppressive capacity. Part of the mechanism leading to this differentiation is mediated via the induction of FOXP3. Phosphorylation of STAT1 Tyr701 following IL-27 stimulation enables its binding to the sites at positions -99bp and -7bp of the FOXP3 promoter, which increases the expression of the *foxp3* gene via epigenetic regulation of the promoter.

2.9 Statement of contribution for publications

I have performed all the experiments for the thesis. I used the FOXP3 promoter into the pGL3 vector that has been previously cloned by Pierre-Yves Mantel as well as the unrelated mutation construct for the directed mutagenesis experiment.

Discussion

By acting directly on chromatin remodeling [156], the transcriptional regulator FOXP3 plays an important role in the early differentiation process of iTreg cells. This thesis revealed a mechanism of FOXP3 expression regulation by Th1 cytokines. In a first step, the influence of Th1 cytokines on iTreg generation was analyzed and led to the finding that IFN-γ and IL-27 further enhance TGF-β-mediated induction of FOXP3. In a second step, the molecular mechanism of the FOXP3 induction was investigated and led to the discovery that following IL-27 costimulation with TGF-β, STAT1 is activated, binds the FOXP3 proximal promoter and permissively modifies the histone acetylation which empowers the FOXP3 expression.

3.1 IL-27 is more than a Th1-inducing cytokine

The cytokine microenvironnement during the priming of naïve $CD4^+$ T cell by APC dictate their differentiation. IL-12, which is produced following type 1 pathogen infection, has been recognized as Th1-inducing cytokine. IL-27 and IFN-γ are necessary, but functionally redundant, for the T-bet and IL-12Rβ2 expression, which increases the IL-12 responsiveness of the naïve cells. However, IL-27 seems to act as a sensor of the current immune state and its effects are flexible according to the type and the amount of cytokines in the milieu. In 3 different inflammatory conditions, IL-27 was observed to act as such sensor. During *Toxoplasma gondii* infection, high levels of IL-12 are present and large amount of IFN-γ are produced. Under this condition, IL-27 does not further enhance Th1 development [149]. However, during a *Leishmania major* infection, at low level of IL-12, or when IFN-γ is neutralized, IL-27 significantly increases IFN-γ production and participates in the Th1 cell differentiation [150]. Following *Trichuris muris* infection, high levels of IL-4 are produced and in this case, the main role of IL-27 is to inhibit the expansion phase of the Th2 cells, mainly by downregulating GATA-3 expression via T-bet [4,162]. IL-27$Ra^{-/-}$ mice develop more severe symptoms in EAE [143,150] showing the ability of IL-27 to directly suppress Th17 cell differentiation. The mechanism by which IL-27 exerts its extensive suppressive effects remains unclear. In this thesis, we suggest that IL-27 potentiates the effect of other cytokines according to the cytokine microenvironment. We show a function of IL-27 in ampliflying the effect of TGF-β on the induction of FOXP3 and thus, in potentiating the induction of iTreg cells. In favour of this hypothesis, our pull down analysis reveals a weak binding of STAT3 to the FOXP3 promoter. STAT1 and STAT3 are known to have opposite function [163] and it was recently shown that STAT3 inhibits iTreg development [164]. IL-6 is a major activator of STAT3 and acts in concert with TGF-β to induce the expression of RORC2, which leads to Th17 cells differentiation [23,24]. Considering the close relationship between Th17 and iTreg cells, IL-27 might play a critical

role in the lineage decision-making between Th17 and iTreg cells. In the presence of TGF-β, IL-27-induced STAT1 might prevent the inhibitory effect of STAT3 binding to the FOXP3 promoter and thus, favouring iTreg differentiation.

3.2 IL-27 increases FOXP3 expression in humans

Costimulation of naïve $CD4^+$ T cells with TGF-β and IL-27 or IFN-γ induces higher FOXP3 expression than TGF-β stimulation alone, and generates potently suppressive iTreg cells. The specific role of IL-27 on FOXP3 expression has been recently investigated in mice and, in contrast to our findings, revealed that IL-27 inhibits TGF-β-driven induction of FOXP3 and iTreg cell differentiation [165,166]. In theses studies, IL-27 was used at lower concentrations than in our study, suggesting that the pleiotropic effects of IL-27 might be concentration dependent. However, the IL-27-mediated induction of FOXP3 was observed in a concentration-dependent manner, even with lower concentrations. We propose that IL-27 play differential role in murine and humans cells. A qualitative species difference for IL-27 has also been suggested regarding its role on monocytic cells. Resting murine macrophages are minimally responsive to IL-27, but human monocytes are strongly activated by IL-27 in a STAT1-dominant manner [167]. Since the function of IL-27 was also STAT1-dependent in the current study, it might explain the these discrepancies between man and mouse.

The equivalence of FOXP3's regulation and function between mice and human is also a matter of debate. While Foxp3 is exclusive to Treg cell lineage in mice, it can be transiently expressed in activated human T cells [110,168,169]. However, the $FOXP3^+$ cells acquire suppressor activity after prolonged cultures [170] and lentivirus-based ectopic expression of FOXP3 is sufficient to generate potent and stable human $CD4^+$ Treg cells [171]. Even though the regulation of FOXP3 expression differs between man and rodents, its expression in human $CD4^+$ T cells relates to regulatory activity.

3.3 IL-27 sustains iTreg differentiation in humans

Since the FOXP3 expression is higher after IFN-γ or IL-27 treatment, we anticipated that iTreg cells driven in the presence of those cytokines would exhibit higher suppressive capacity. However, the proliferative response of co-cultured $CD4^+$ responder T cells was similar between all Th1-boosted iTreg and regular iTreg cells. Since post-translational modifications of FOXP3 influence its function *in vivo* [172], additional factors modifying FOXP3's acetylation or phosphorylation may be required to translate expression differences into higher suppressive functionality.

Together with the surface expression of distinctive Treg cell markers, the IL-27-treated iTreg cells are capable of producing the newly identified Treg cytokine IL-35. Even though it the exact function of this cytokine in the human system remains to be elucidated, these findings support the regulatory phenotype of the iTreg cells cultured in the presence of TGF-β and IL-27.

3.4 T-bet is a secondary player in the induction of FOXP3

Because naïve $CD4^+$ T cell differentiation is orchestrated by lineage-specific factors, we anticipated that the Th1-specific factor T-bet would inhibit the differentiation of Treg cell subsets like it was reported concerning Th2 cells differentiation. T-bet was shown to repress Th2 lineage commitment through tyrosine kinase-mediated interaction with GATA-3, that interferes with the binding of GATA-3 to its target DNA [168]. However, the positive correlation between T-bet and FOXP3 expression, and the early increase of T-bet following cell stimulation, suggest a role for T-bet in inducing FOXP3. GATA-3 has been shown to inhibit FOXP3 expression via direct interaction to the FOXP3 promoter [136]. The tyrosine kinase-mediated interaction between T-bet and GATA-3 may reduce the GATA-3-mediated inhibition on the *foxp3* gene, and thus favour FOXP3 expression.

In addition to GATA-3's capacity to induce Th2 cytokine production, it can block Th1 cytokine production via down-regulation of STAT4 and indirectly the IL-12Rβ2 chain [10]. However, if T-bet is induced at sufficient levels, such GATA-3 suppression can be counteracted. This ability of T-bet to oppose the action of GATA-3 may be not only essential in permitting Th1 differentiation, but also in favouring iTreg cell differentiation, particularly in the presence of IL-27.

3.5 IFN-γ acts in an indirect manner

In addition to IL-27, IFN-γ also potentiates TGF-β-mediated induction of FOXP3. Even though the neutralisation of IFN-γ diminished TGF-β-induced FOXP3 expression, the induction of FOXP3 was not in a concentration dependent manner. This suggests an indirect effect of IFN-γ on FOXP3. Furthermore, pull down analysis of STAT1 using IFN-γ stimulated $CD4^+$ T cells failed to show any STAT1 binding to the FOXP3 promoter (data not shown). The duration of STAT activation has been associated with the magnitude of target gene expression [169-171]. In the presence of TGF-β, we showed that tyrosine phosphorylation of STAT1 is sustained for a long time following IL-27 stimulation, but waned more rapidly with IFN-γ stimulation. The long lasting effect of IL-27 on STAT1 phosphorylation can be attributed to the WSX1 subunit of its receptor. It has been shown to activate STAT1 in other systems [172]. The gp130 subunit of the IL-27R functions as a signal transducer and as a target for inhibition. Activation of STAT1 by IFN-γ is resistant to inhibition by TLR-induced pathways, whereas IL-27 activated STAT1 is susceptible to inhibition by factors activating p38. Thus STAT1 is differentially regulated following IL-27 or IFN-γ stimulation and only the IL-27-induced STAT1 is implicated in FOXP3 induction. Possibly to bypass this impaired STAT1 function, both IL-27 subunits are induced following IFN-γ treatment. This suggests that induction of FOXP3 by IFN-γ is a subsequent effect of IL-27.

3.6 IL-27-induced STAT1 regulates foxp3 gene expression

It is well established that TGF-β induces FOXP3 expression and can convert $CD4^+CD25^-$ into $CD4^+CD25^+$ Treg cells. Until very recently, no molecular mechanisms had been proposed to explain the manner by which TGF-β mediates FOXP3 induction. It was suggested that TGF-β is mandatory for the induction of FOXP3 by keeping a low expression of GATA-3 and T-bet [173-175]. TGF-β mediates the phosphorylation of intracellular Smad proteins, which enables the association of Smad4, Smad2 and Smad3. After translocation into the nucleus, Smad complexes bind to Smad binding element (SBE) and control gene expression by recruiting coactivators that contain HAT activity or corepressors with HDAC activity to activate or repress target genes, respectively [176]. Recently, a SBE site was discovered in the enhancer region located downstream of the *foxp3* gene's TSS [120]. The transcription factors Smad3 and NFAT are required for activity of this Foxp3 enhancer, and both factors are essential for histone acetylation in the enhancer region. This allows the enhancer complex to interact with the promoter region and to induce Foxp3 expression. We previously showed that the FOXP3 promoter region carries an important regulatory unit for the regulation of the *foxp3* gene. The FOXP3 human promoter was previously localized by 5'-RACE, at -6221 bp upstream from the TSS [122]. The sequence upstream of the UTR shows a high degree of conservation between human, mouse and rat, which indicates the presence of important regulatory elements. In addition to several NFAT and AP-1 binding sites that act as TCR-responsive units of the *foxp3* gene, the FOXP3 promoter contains many GATA-3 binding sites that negatively regulate FOXP3 expression. In this thesis, we identified 2 functional STAT binding sites. TESS analysis predicted 4 putative STAT binding sites (TTN_5AA) within the region -511/+177 of the human FOXP3 promoter (Site 373bp, Site 351bp, Site 99bp and Site 7bp upstream the TSS). Consistent with previously demonstrated STAT1-

mediated effect of IL-27 on naïve T cell differentiation, we show that IL-27 regulates the induction of the human *foxp3* gene through the activation of the STAT1 transcription factor. An important role of STAT1 in regulating immune response has previously been shown. STAT1-deficient mice expressing a transgenic TCR against myelin basic protein have higher susceptibility to EAE due to a reduced number as well as a functional impairment of the $CD4^+$ $CD25^+$ Treg cells [151]. Even though the determination of Treg cells was based only on the CD25 expression and not on FOXP3 expression, the adoptive transfer of wild-type Treg cells into STAT1-deficient hosts was sufficient to prevent the development of autoimmune disease. This demonstrates an essential role of STAT1 in the maintenance of immunological self-tolerance. By binding to the proximal part of the promoter, STAT1 directs the level of transcription of the *foxp3* gene.

3.7 IL-27 regulates FOXP3 expression at the epigenetic level

Chromatin opening is an important step in de-repressing silenced genes [177,178] and the expression of the *foxp3* gene depends on the availability of its chromatin [124,179]. Permissive histone modifications of the FOXP3 promoter, the intronic differentially methylated region 3 and the enhancer at position +2079 to +2198 were reported in Treg cells [120,124,179]. The histone modifications near the *foxp3* TSS were described as a marker of the inducibility of Foxp3 and correlate with its expression [180]. STAT1 has been described to induce transcription by chromatin remodelling following IFN-γ stimulation [155]. In this thesis, we confirm that histone acetylation of this region promotes FOXP3 expression and reveal that this mechanism is mediated by IL-27-induced STAT1. The late increase of FOXP3 following TGF-β stimulation implies the need of numerous intracellular mechanisms to regulate its expression. The rapid binding of STAT1 to the FOXP3 promoter argues that IL-27-induced STAT1 may regulate early events allowing the recruitment and binding of the other regulatory factors (e.g. NFAT, AP-1, Sp-1) to empower *foxp3* gene transcription.

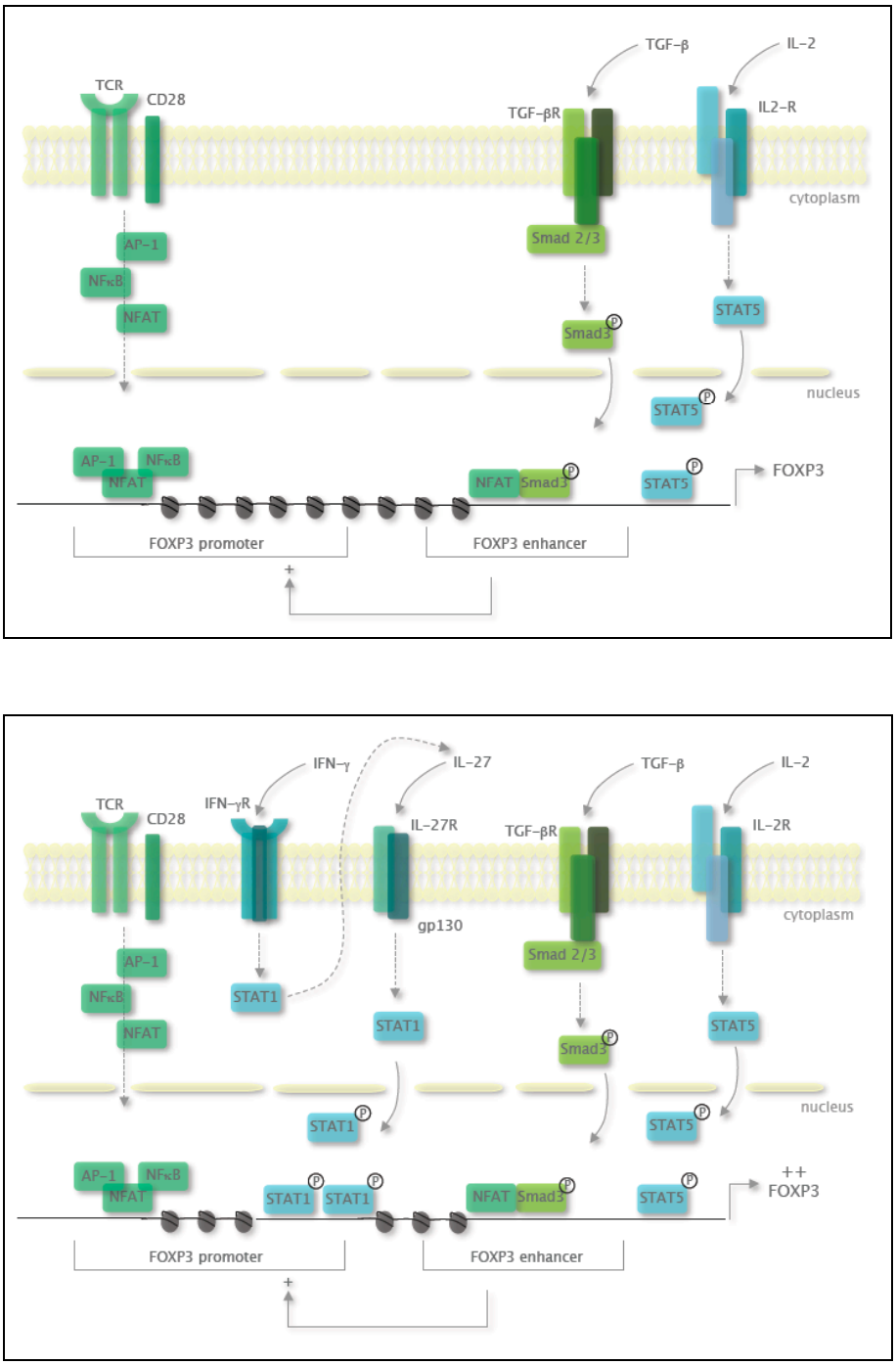

Figure 28: Model of FOXP3 regulation by Th1 cytokines

The upper scheme represents the *foxp3* gene regulation by TCR, TGF-βR and IL-2R stimulation. The chromatin of the proximal promoter is in a restrictive conformation. The bottom scheme depicts the effect of IFN-γ and IL-27 stimulation. IFN-γ might participate indirectly on FOXP3 induction via the production of IL-27. Activation of the gp130 subunit of the IL-27R has a long lasting activating effect on STAT1, which allows its binding to the proximal part of the promoter. This binding increases the accessibility of the chromatin, which further increases the FOXP3 expression levels.

3.8 Conclusion and Outlook

By investigating the human FOXP3 promoter we could explain the molecular mechanisms involved in FOXP3 up-regulation following costimulation of naïve $CD4^+$ cells by TGF-β and IL-27 or IFN-γ. We also described that IL-27-induced STAT1 acts as an amplifier of FOXP3 expression that binds directly and represses the FOXP3 promoter, which increases the accessibility of the chromatin (Figure 28).

Our data suggest a new pathway of regulation for FOXP3 induction with permissive epigenetic modification of the proximal promoter by IL-27-induced STAT1 molecule. The mechanisms involved in regulating FOXP3 in iTreg cells are of considerable interest due to the essential contribution of FOXP3 in preventing autoimmune diseases. The results of this thesis not only gives new insights into the molecular mechanisms implicated in regulating FOXP3 expression and iTreg generation, but may also lead to the development of therapeutic approaches targeting IL-27 in order to modulate iTreg induction or to restore tolerance in many immune-mediated diseases.

Curriculum Vitae

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4.2 Education

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May 2003 - May 2005	*University of Montreal, Research Centre Sacre-Cœur Hospital, Montreal, Canada.* **Master of Science** (M.Sc.) in **Microbiology and Immunology**. Director: Dr. Karim Maghni. Title of the project: Regulation of the modulation of basophil functions by tachykinins.
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health.

August 1998 - May 2000 *Cegep André-Laurendeau, Montreal, Canada.* **International Baccalaureate** (I.B.) in **Health sciences**.

4.3 Courses during PhD

July 20th-27th 2007 RCAI International Summer Program 2007. Yokohama, Japan.

November 16th-18th 2006 European Medical Writer Association, Autumn Conference, Brussel, Belgium.

4.4 Journal Clubs

Ouaked, N. Journal Club at the Swiss Institut for Allergy and Asthma Research. "Smad 3 and NFAT cooperate to induce Foxp3 expression through its enhancer". Davos, February 2008.

Ouaked, N. Journal Club at the Swiss Institut for Allergy and Asthma Research. "Nonself-antigens are the cognate specificities of $FOXP3^+$ regulatory T cells". Davos, October 2007.

Ouaked, N. Journal Club at the Swiss Institut for Allergy and Asthma Research. "Conventional T-bet^+FOXP3^- Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection". Davos, March 2007.

Ouaked, N. Journal Club at the Swiss Institut for Allergy and Asthma Research. «Role of IFN-γ in induction of Foxp3 and conversion of $CD4^+CD25^-$ T cells to $CD4^+$ Tregs ». Davos, September 2006.

Ouaked, N. Journal Club at the Swiss Institut for Allergy and Asthma Research. « TGF-β in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells». Davos, April 2006.

Ouaked, N. Journal Club at the Swiss Institut for Alelrgy and Asthma Research. « Basophils play a critical role in the development of lgE-Mediated Chronic Allergic Inflammation Independently of T cells ans Mast Cells ». Davos, October 2005.

4.5 Progress Reports

Ouaked, N. Progress report at the Swiss Institut for Allergy and Asthma Research. "The role of Th1 cytokines on the induction of FOXP3". Davos, February 2008.

Ouaked, N. Progress report at the Swiss Institut for Allergy and Asthma Research. "IL-27 and IFN-γ induced STAT1 has a role in FOXP3 induction". Davos, November 2007.

Ouaked, N. Progress report at the Swiss Institut for Allergy and Asthma Research. "STAT implication in the regulation of FOXP3". Davos, April 2007.

Ouaked, N. Progress report at the Swiss Institut for Allergy and Asthma Research. "*Role of Th1 specific elements in Tregs induction*". Davos, October 2006.

Ouaked, N. Progress report at the Swiss Institut for Allergy and Asthma Research. "*Role of Th1 specific elements in Tregs induction*". Davos, July 2006.

Ouaked, N. Progress report at the Swiss Institut for Allergy and Asthma Research. *« The role of IL-27 on T regulatory cells induction*». Davos, November 2005.

4.6 Promotion's Komitee Meeting

Ouaked, N. Progress report at the Swiss Institut for Allergy and Asthma Research. *« The role of IL-27 on T regulatory cells induction*». Davos, November 2005.

Ouaked, N. Progress report at the Swiss Institut for Allergy and Asthma Research. *« The role of IL-27 on T regulatory cells induction*». Zürich, December 2006.

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Ouaked, N., Mantel, PY., Bassin, C., Burgler , S., Siegmund, K., Akdis, CA,. Schmidt-Weber, CB. (2008) "Regulation Of The *Foxp3* gene By The Th1 Cytokines: The Role Of IL-27-Induced STAT1" In revision in J Immunol.

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Meloche, C., Lefort, B., Poirier, A., **Ouaked N**. and Maghni, K. (2004) "Role of Neurokinins in $CD4^+$ T Cells Apoptosis: Determination of Neurokinin-1 Receptor and Substance P Expression in Jurkat T Cells." Immunology 2004. Supplement presented at the 12^{th} Int. Congress of Immunology and 4^{th} annual Conference of FOCIS (Montreal, Canada, July 18-23, 2004), 479-483.

Congress Attendance

6.1 Poster Presentations

Ouaked, N., Mantel, P-Y., Rückert, B., Bassin, C., Siegmund, K., Akdis, C.A. and Schmidt-Weber, C.B. "The regulation of FOXP3 by Th1 cytokines". World Immune Regulation Meeting 2. Davos, Switzerland, March 17th-20th 2008.

Ouaked, N., Mantel, P-Y., Rückert, B., Bassin, C., Siegmund, K., Akdis, C.A. and Schmidt-Weber, C.B. "IL-27 induced STAT1 has a role in T regulatory cells induction". RCAI International Summer Program 2007. Yokohama, Japan, July 20th-27th 2007.

Ouaked, N., Mantel, P-Y., Rückert, B., Bassin, C., Siegmund, K., Akdis, C.A. and Schmidt-Weber, C.B. "IL-27-induced STAT1 plays a role in FOXP3 induction". World Immune Regulation Meeting I. Davos, Switzerland, April 11th-15th 2007.

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Ouaked, N., Mantel, P-Y., Rückert, B., Bassin, C., Siegmund, K., Blaser, K. and Schmidt-Weber, C.B. "IL-27 : The link between Th1 and T regulatory cells". 4th EAACI GA^{2}LEN Davos Meeting. Garmisch-Partenkirchen, Germany, February 16-19, 2006.

6.2 Oral presentations

Ouaked, N., Mantel, P-Y., Rückert, B., Bassin, C., Siegmund, K., Akdis, C.A. and Schmidt-Weber, C.B. 'The influence of T helper 1 cells related cytokines on the generation of inducible T regulatory cells' 6th EAACI-GA2LEN Davos Meeting. "Basic Immunology Research in Skin Allergy and Immunotherapy". Hotel Pichlmayrgut, Pichl/Schladming, Austria, January 31st-February 2nd.

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Ouaked, N., Mantel, P-Y., Rückert, B., Bassin, C., Siegmund, K., Akdis, C.A. and Schmidt-Weber, C.B. "The influence of T helper 1 cells specific elements on the induction of T regulatory cells" 5th EAACI GA2LEN Davos Meeting, "Basic Immunology Research in Allergy and Clinical Immunology" Sunstar Park Hotel, Davos, Switzerland, February 1st-4th, 2007

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